

Molecular characterisation of the sheep scab mite,
Psoroptes ovis

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DECLARATION

The work carried out in this thesis is my own original work, except where otherwise stated, and it has not been submitted for any other degree or professional qualification.

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ABSTRACT

Sheep scab is a highly contagious ectoparasitic disease that is caused by infestation with the mite, *Psoroptes ovis* (Acari: Psoroptidae). The disease is an important welfare issue due to the clinical symptoms induced, which include intense pruritis and severe exudative dermatitis. The host response is typical of an immediate hypersensitivity reaction however, there has been little definition of the antigens present in the mites to date. The main aims of this study were to identify the allergens associated with the sheep scab mites, and to determine the main antigens present.

The proteases present in the water-soluble (S1) and membrane associated (S2) *P. ovis* extracts were characterised because of their allergenic potential by analogy with other organisms, particularly house dust mites. This was conducted using a combination of substrate gels and protein degradation assays. Substantial protease activity was present with cysteine proteases dominating, although aspartyl and metallo-proteases were also present. These proteases were found to degrade many substrates found in the mites' natural habitat, e.g. gelatin, collagen, fibronectin, and haemoglobin. The proteases were not inhibited by antibody from animals with a current infestation, however, this does not rule out the possibility that these proteases are targets for the host immune response.

A complementary DNA (cDNA) library was prepared from mixed stage mites in a lambda TriplEx2 vector, for the dual purpose of immunoscreening and expressed sequence tag (EST) analysis. Immunoscreening was carried out using sera from animals which had been immunised and protected against mite challenge with mite extracts and 48 cDNA clones were selected. Of these, six were recognised in a differential immunoscreen using sera from strongly protected animals compared with sera from weakly protected animals. Subsequent sequence analysis established that these clones had homology to myosin or myosin-based proteins.

The EST analysis of 500 randomly selected cDNA clones identified many novel proteins and this study focused attention on the allergens, proteases and antioxidant enzymes. Eight different allergens were identified including homologues of the major house dust mite (*Dermatophagoides* spp.) allergens. The group 2

allergens, e.g. *Der f 2*, were most abundant in the EST dataset. Cathepsin B and L cysteine proteases were identified as well as several antioxidant enzymes, e.g. glutathione S-transferase, superoxide dismutase and thioredoxin peroxidase, which may help to protect the mite from toxic free radicals released in the host lesion.

This study has shown that there are a range of proteases present in *P. ovis* mites. Eight putative allergens and three classes of antioxidant enzymes were also identified. These results provide an insight into the physiology of the mite, and the aetiology of the disease.

ABBREVIATIONS

AChE	acetylcholinesterase
AEBSF	4-(2-aminoethyl) benzene sulfonyl fluoride
APS	ammonium persulphate
bp	base pairs
°C	degrees Celcius
cDNA	complementary deoxyribonucleic acid
cm	centimetre
DAB	3,3' diaminobenzidine
dH ₂ O	distilled water
dNTP	deoxynucleotide
ddNTP	dideoxynucleotide
DTT	dithiothreitol
DNA	deoxyribonucleic acid
DNTB	dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
E/S	excretory/secretory
EST	expressed sequence tag
E64	L-trans-epoxysuccinyl-L-amido-(4-guanidino)-butane
g	grams
<i>g</i>	gravitational force
GPX	glutathione peroxidase
GST	glutathione S transferase
hr	hour
HRP	horse radish peroxidase
Ig	immunoglobulin
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kb	kilobases
kDa	kilo Dalton
kg	kilogram

L	litre
M	molar
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetre
MMLV	monkey murine leukaemia virus
mRNA	messenger ribonucleic acid
nm	nanometre
µg	microgram
µl	microlitre
µM	micromolar
OD	optical density
OP	organophosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pH	-log ₁₀ (hydrogen ion concentration)
pmol	picomoles
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcriptase
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
spp.	species
SP	synthetic pyrethroid
S1 extract	water soluble fraction of <i>P. ovis</i> mites
S2 extract	membrane associated fraction of <i>P. ovis</i> mites
TAE	tris-acetic acid-EDTA buffer

TBS	tris buffered saline
TBST	TBS with Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)amino methane
Triton X-100	octylphenoxypolyethoxethanol
U	unit
UV	ultraviolet
V	volt
W	watt
w/v	weight per volume
v/v	volume per volume
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

Sheep scab has been recognised since biblical times as a debilitating disease of sheep. In 1807, it was recognised that a mite was the causative agent of this disease. The mite was named *Psoroptes communis ovis* by Hering (1838) and its lifecycle described by Gerlach in 1857 (cited in Henderson, 1991). The mites have a worldwide distribution, infecting sheep, cattle and goats. Sheep scab is a highly contagious disease that is most common in sheep in the UK and in cattle in Europe and the USA. It is considered an important welfare issue due to the symptoms of disease, which include intense pruritis and exudative dermatitis.

Early control measures included lime, sulphur and arsenicidal compounds, which were poured onto the skin. Plunge dipping was first used in the early 1800's, and the national compulsory dip was introduced in 1906 (Kirkwood, 1985). By 1914, it was recognised that the compounds were only effective if used twice, fourteen days apart and in 1926 it was realised that, to be fully effective, the acaricide had to be in contact with the fleece for a full minute. In 1948, a new compound, gamma benzene hexachloride (gamma hexachlorocyclohexane (HCH) or lindane), revolutionised the dipping process, as it was effective after a single dip (Henderson, 1991). This was a highly successful product and, in 1952, sheep scab was eradicated from the UK. However, the disease was reintroduced from animals imported from Ireland in 1973 and since then its prevalence has been increasing throughout the UK. Over the 20 years from 1973 until deregulation in 1992, a total of just under 1,500 flocks were reported to have sheep scab. The number of cases reported each year reached a peak in 1983, when approximately 150 new flocks were reported to have the disease (French, Berriatua, Wall et al., 1999). The prevalence of the disease is thought to have increased since deregulation in 1992 (Coles, 1998). At present, the actual number of cases of sheep scab in the UK is unknown, although it has been reported from most counties of England and Wales and is estimated to affect between 500-10,000 farms (M. Taylor, personal communication). Most of the other countries with sheep scab have had similar results as the UK, whereby the

disease has been eradicated at some point only to be re-introduced at a later date. The only countries that have managed to eradicate the disease successfully and remain free to this day are Australia and New Zealand. Control of the disease at present is through chemical intervention, although the emergence of resistant strains of mite and concerns over associated health and environmental risks suggest that this strategy will not be effective in the long term.

1.1.1 Phylogeny

Sheep scab mites are astigmatid mites of the family Psoroptidae, in the subclass Acari. There are four genera described in the family Psoroptidae. *Psoroptes* species (spp.) are the scab mites and are found mainly on sheep or cattle, *Chorioptes* spp. mites are generally found on the legs and under body of ungulate herbivores, *Otodectes* spp. parasitise the ears of carnivores and *Caparinia* spp. are associated with the European hedgehog (Bates, 1999b).

Psoroptes spp. are non-burrowing, obligate ectoparasites of mammals with a cosmopolitan distribution. Adult female *P. ovis* mites are slightly larger than the males and are approximately 0.75 mm in length. The legs of *P. ovis* mites project beyond the body margin and terminate in funnel-shaped suckers (pulvilli); this distinguishes *Psoroptes* mites from other genera in the family, which have bell-shaped pulvilli (Urquhart, Armour, Duncan et al., 1987; Bates, 1999a). Although the mites appear white in colour on sheep, on other hosts they may appear dark red or black. Wright & DeLoach (1981) suggested that this was due to the ingestion of red blood cells, whereas Rafferty & Gray (1987) suggested that, as mites develop, they become darker in colour as a result of changes in the internal organs.

Although the classification of *Psoroptes* mites has been subject to a great deal of change the results of an extensive morphological study carried out by Sweatman (1958) provide the basis for what is widely regarded as the true classification. The study compared the host species parasitised, location of the mites on the host and the length of the outer opisthomal setae on adult male mites. After comparison of these morphological characteristics, Sweatman concluded that there are five species: the body mites *P. ovis*, *P. equi* and *P. natalensis*, and the ear mites *P. cuniculi* and *P. cervinus*.

Differentiation between the sheep body mite, *P. ovis*, and the rabbit ear mite, *P. cuniculi*, has recently been the subject of much debate. Many people regard these mites as two different subspecies. However, *P. ovis* and *P. cuniculi* are morphologically similar, can infect and produce reproductive colonies on sheep (Bates, 1991) and cattle (Wright, Riner, & Guillot, 1983) and ingest similar quantities of haemoglobin when feeding from rabbits (Rafferty & Gray, 1987). In addition, the second internal transcribed spacer of the ribosomal RNA in these species are highly homologous so they are regarded as being conspecific (Zahler, Essig, Gothe et al., 1998). For these reasons, it is now considered that *P. ovis* and *P. cuniculi* are variants of the same species that inhabit different niches (Bates, 1991; Bates, 1997a). In fact, a recent paper suggested that the original name of *P. communis* should be re-instated as the species name, and the variants named *P. communis* var. *ovis* or *P. communis* var. *cuniculi* (Bates, 1999b). However, Zahler, Hendriks, Essig et al. (2000) suggested that the main species name should be *P. equi* as it was the first *Psoroptes* species described (Hering 1838, cited in Zahler et al., 2000), and that all the other species should be synonyms of this species. Evidently, the debate on the species classification of the psoroptic mites has not been resolved.

1.1.2 Lifecycle

The lifecycle of *P. ovis* mites has been described by a variety of authors, each using their own terminology for the various stages (Shilston, 1915; Downing, 1936; Sweatman, 1958). However, it now appears to be widely agreed that the lifecycle consists of 5 stages of development, the egg, larva, protonymph, tritonymph, and adult (Figure 1.1, Sanders, Froggat, Wall et al., 2000). The lifecycle takes approximately 14 days to complete and all stages occur on the sheep (Kirkwood, 1986). The eggs are laid close to the surface of the skin and take approx. 3 days to hatch (Downing, 1936). The larval stage initiates feeding immediately after hatching and feeds for between 2 and 3 days. Larvae then enter a non-feeding quiescent phase lasting 12-48 hours, during which ecdysis occurs. During the quiescent phase, larvae are motionless and in a characteristic position, with all the legs stretched forward beyond the body margin. The protonymph then emerges and feeds for 24-72 hours before undergoing a further quiescent stage and ecdysis to emerge as the tritonymph.

Adult males attach to the tritonymph female, but copulation only occurs when the female has moulted to the adult stage (Guillot & Wright, 1983). An adult female ovigerous mite has a life span of approx. 40 days, and may lay 1-2 eggs per day (Kirkwood, 1986; Bates & Groves, 1991).

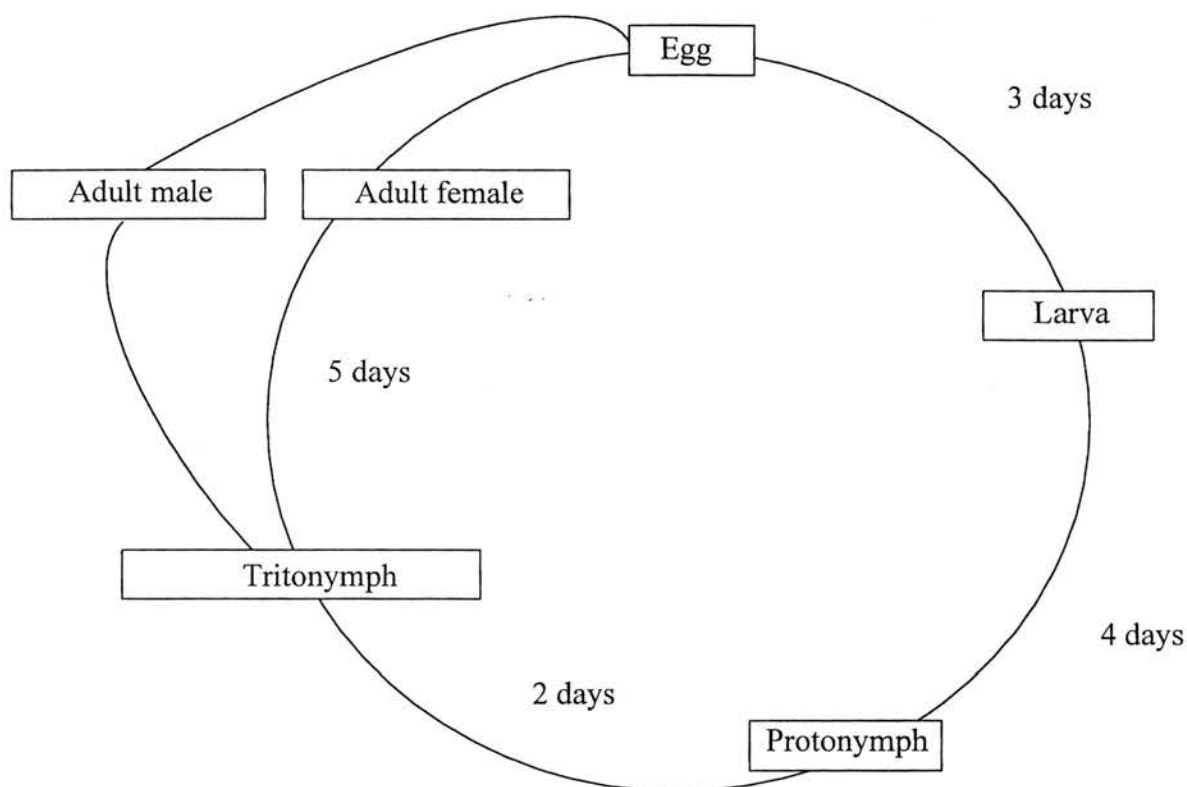


Figure 1.1: Lifecycle of *P. ovis* mites

1.1.3 Seasonal Variation in Disease

Initially the disease was thought to be a strictly winter condition, with the mites having a latent period in cryptic sites over the summer. Downing (1936) suggested that the mites migrate to the ears, inguinum and the digital and orbital fossae throughout the summer months, returning to the body during the winter. This theory was widely accepted and led to complacency that the disease would not occur throughout the summer. As a result, many people failed to recognise cases of sheep scab that occurred during the summer months. However, in 1983 a review by Kirkwood suggested that the mites do not have a latent phase in the summer months,

but actually become more active at this time. Blachut, Roberts, & Meleney (1973) observed that mites of all stages were present throughout the summer, although the number of lesions was smaller. They also found that the mites (regardless of the stage of development) did not have a preferred season. No migration was found to occur and the mites reached the supposed latent sites only when the primary lesion had reached these areas (Kirkwood, 1985). Therefore, the current understanding of the disease regards sheep scab mites as active all year round, so that sheep are susceptible to infestation at any time of the year.

1.1.4 Transmission

The transmission of sheep scab generally occurs through direct contact. However, other methods of transmission are possible. For example, trailers, bedding and wool tags are thought to be viable sources of infective mites (Lewis, 1997). Wilson, Blachut, & Roberts (1977) found that naïve animals could acquire mites from sheep pens up to 72 hours after the pens had been vacated by *P. ovis*-infected animals. Although other infested host species may transfer the infection or act as a temporary reservoir of *P. ovis*, there are conflicting reports on this subject. Experimental transmission of mites has been successful in a number of experiments. For example, transmission of *P. cuniculi* from rabbits to rabbits, sheep, cattle and goats and transmission of *P. ovis* from sheep to goats, cattle and rabbits was successful (reviewed Zahler et al., 2000). However, experimental transmission of *P. ovis* from sheep to cattle, goats and sheep, or transmission of *P. cuniculi* from rabbits to sheep or cattle was unsuccessful in the hands of other authors (reviewed Zahler et al., 2000). Nonetheless, natural transmission of psoroptic mites has been found to occur, for example from sheep to sheep and cattle (Roberts & Meleney, 1971), although this was not effective in the transfer of mites from either infected sheep to cattle or goats, or from infested cattle or goats to sheep (Evans & Kirkwood, 1984; O'Brien, Gray, & O'Reilly, 1994a). Sheep are thought to be the only reservoir of *P. ovis* in the UK because, although psoroptic mange has been identified in domestic rabbits, no infection has been found in the wild rabbit population. Also, there have been very few cases of psoroptic mange in cattle, and each of these cases has been

shown to have been imported from Europe, where the disease is much more common (Evans & Kirkwood, 1984; Kirkwood, 1985).

1.1.5 Survival off the Host

The length of time that the mites can remain infective when off the host has been the subject of much debate. For example, although mites were thought to remain infective for up to 2 years when removed from the host (Salmon & Stiles, 1903), Tarry (1974) concluded that the maximum was only 3 days. Different conditions were used to house the mites in each of these experiments. Recently however, mites exposed to normal outdoor conditions of temperature and humidity were found to remain infective for up to 14 days off the host (O'Brien, Gray, & O'Reilly, 1994b). The same authors found that mites implanted onto experimental animals after 16 days off the host failed to cause clinical disease. The energy reserves held by the mites after 16 days off the host may not withstand the requirements needed to infest a new host. Also, any females present may have already laid their eggs and, without food, the juveniles would not develop properly.

1.1.6 Feeding

To understand the feeding mechanisms of the *P. ovis* mites, one must understand the environment on which they live. The mites are found on the surface of the host skin and, unlike some other species of mites, they do not burrow through the skin. All skin has the same basic structure (Figure 1.2) with three easily identifiable layers, the loose stratum corneum, the intact stratum corneum and the uncornified epidermis (Lloyd, Amakiri, & McEwan Jenkinson, 1979). There is also an additional layer present above the loose stratum corneum composed mostly of lipid.

Initially, it was thought that the mites pierced the skin to feed (Shilston, 1915). However, later studies suggested that mites did not require to pierce the skin and an *in vitro* experiment showed that the mites can suck serous fluid by the means of a pseudorutellae (Blake, Bay, Meola et al., 1978). Rafferty & Gray (1987) studied the mouthparts of *P. ovis* and *P. cuniculi* mites and found that they are suited to both fluid and solid feeding. They suggested that the lobes of the hypostome may be used to abrade the skin surface, and then transfer the fluid to the pre-oral cavity. Sinclair

& Kirkwood (1983) found no evidence that the mites penetrate past the outermost stratum corneum and so the mites are now believed to browse on the skin surface.

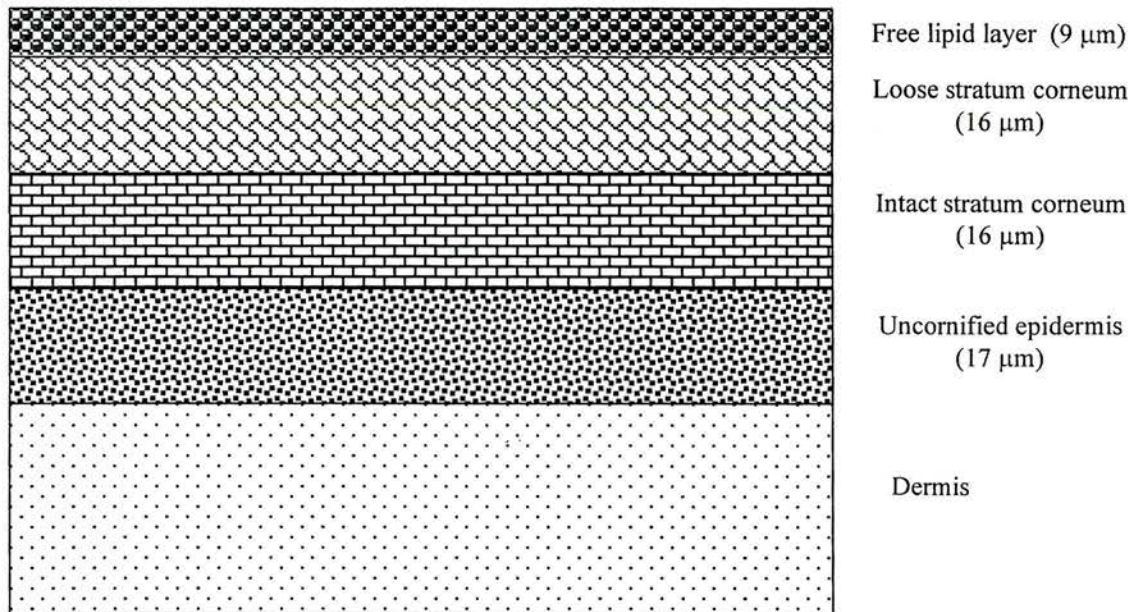


Figure 1.2 Schematic diagram of a vertical section through sheep skin. This diagram was adapted from Lloyd, Amakiri, & McEwan Jenkinson (1979).

The mite digestive system is composed of a foregut, midgut, and hindgut. In *Psoroptes* mites, the foregut and hindgut are cuticle-lined and the midgut is lined with microvilli, suggesting that the absorption of the nutrients occurs mainly in the midgut (Mathieson, 1995; Mathieson & Lehane, 2002). The mid-gut can be divided into three distinct areas, the stomach, colon and post-colon. Faecal material is thought to be surrounded by a peritrophic membrane (Mathieson, 1995; Mathieson & Lehane, 2002), as has been identified in other mite spp., for example *Dermatophagoides* spp. (house dust mites; Wharton & Brody, 1972; Brody, McGrath, & Wharton, 1972). The peritrophic membrane can be defined as an acellular lining found in the gut lumen that separates ingested food from the gut epithelium (Eisemann & Binnington, 1994). The origin of the peritrophic membrane in *P. ovis* has not yet been identified, although it is first detected in the post-colon (Mathieson & Lehane, 2002).

Sinclair & Kirkwood (1983) detected the presence of lipid molecules in the mite body cavity and therefore suggested that the mites feed superficially on the outer layers of the skin. There is a continuous layer of lipid on the surface of healthy sheep skin (approx. 9 μm thick) that is not present on cattle (Lloyd, Amakiri, & McEwan Jenkinson, 1979) and this may serve as the primary food source during early infestation (Sinclair & Filan, 1989). Recent enzymatic studies carried out on *P. ovis* may provide further evidence for this theory, as lipase activity was identified (Nisbet & Billingsley, 2000). Many skin-dwelling bacteria are also present on the skin surface (Murray & Edwards, 1987), and these may provide an alternative food source (Sinclair & Filan, 1989). However, more recent studies show that some of the bacteria identified from *P. ovis* are *Serratia marcescens* (Mathieson & Lehane, 1996), which are not normally present in sheep skin (Murray & Edwards, 1987; Jansen & Hayes, 1987). Some authors have suggested that haemoglobin may be another food source for the mites, and indeed haemoglobin has been detected from mites that have fed on rabbits (Wright & DeLoach, 1980; Rafferty & Gray, 1987), and cattle (Wright & DeLoach, 1981), but not from sheep (Rafferty & Gray, 1987). An experiment by DeLoach (1984) found that *P. ovis* mites will choose to feed on water, plasma or serum, and a low-salt diet in preference to whole blood. Recent studies only identified host eosinophils in the mites, as opposed to lipid or bacteria (Mathieson & Lehane, 2002). The authors suggest that the mites do not feed on epithelial debris or blood, but instead feed on serous fluid released by the inflamed skin of infested animals.

1.2 IMMUNOPATHOGENESIS

Immune reactions between ectoparasites and their hosts have been extensively studied in tick systems. Ticks stimulate many immune responses in their hosts including tick-specific antibody and cytokine production and itch sensations (Wikel & Bergman, 1997; Brossard & Wikel, 1997; Wikel & Alarcon-Chaidez, 2001). For example, histamine derived from mast cells and basophils inhibits tick salivation and engorgement (Brossard & Fivaz, 1982). To combat these responses ticks have developed countermeasures such as suppression of the hosts innate and acquired immune responses (Wikel & Bergman, 1997; Wikel & Alarcon-Chaidez,

2001). Hosts that have acquired resistance to ticks show grossly different cutaneous reactions to tick feeding compared to susceptible animals. For example, resistant hosts have an influx of basophils and eosinophils at the attachment sites, which suggest a role of a form of delayed type hypersensitivity known as cutaneous basophil hypersensitivity (Allen, 1989). Cutaneous basophil hypersensitivity is also observed in sheep with *P. ovis* infestation (van den Broek, Huntley, Machell et al., 2000). The work carried out on the ticks, and the recent studies on *P. ovis*, suggest that there may be many complex reactions between the host and *P. ovis* mites. As the mites are obligate ectoparasites, it may be expected that they have developed many mechanisms to counter the host immune responses.

At present little is known about the molecular mechanisms of resistance to *P. ovis* infestation in sheep and the interactions between mite and host, although work is currently underway. Sheep scab is thought to be a form of allergic dermatitis, due to a host reaction to antigenic material (Sinclair & Kirkwood, 1983) secreted or excreted by the mites (Bygrave, Bates, & Daniel, 1993). In the closely related species, the house dust mites, *Dermatophagoides* spp., the antigenic material is thought to be released in the mite faeces (Tovey, Chapman, & Platts-Mills, 1981).

The host immune reaction to *P. ovis* infestation is typical of an antibody mediated type 1 hypersensitivity response (Stromberg & Fisher, 1986; Losson, Detry-Pouplard, & Pouplard, 1988). This response has been studied in both sheep and cattle and manifests as an acute cutaneous inflammatory reaction (van den Broek et al., 2000). The cutaneous inflammatory response is characterised by an influx of eosinophils, increase in circulating eosinophilia, distinct cell hyperplasia and oedema within the dermis (Stromberg, Fisher, Guillot et al., 1986; Stromberg & Fisher, 1986; van den Broek et al., 2000). A cutaneous basophil hypersensitivity was also identified in sheep (van den Broek et al., 2000). Following intradermal injection of soluble *P. ovis* antigens in unstanchioned cattle, an immediate hypersensitivity and a delayed type reaction were observed (Losson, Detry-Pouplard, & Pouplard, 1988). The clinical symptoms of intense inflammation and oedema in the epidermis may bring about many changes in the epithelium such as capillary dilation and endothelial hypertrophy (Stromberg & Fisher, 1986). These changes may induce a reduction in the barrier function in the skin, increasing the leakage of fluid, protein and other

cellular components from the skin resulting in the presence of serous exudate throughout the course of infection.

In the initial stages of disease, small focal lesions develop at the site of infestation. As the disease develops, the lesions expand and the exudate dries to form the characteristic yellow scabs. New lesions then spread from the original site of infection, with the disease reaching peak severity in 8-12 weeks. Generally, the mites spread over the sheep only as the scab spreads, and mites are not found in the dry centre of the lesion (Kirkwood, 1985). The clinical symptoms observed in sheep and cattle are very similar, although there are a few differences. For example, mites are found only at the edges of the lesion in sheep, whereas they are found throughout the entire lesion in cattle. This is thought to be due to differences in the lesion, the sheep lesion dries out fairly quickly to form a dry centre, whereas the cattle lesion retains moisture, probably maintaining a suitable habitat for the mites (Kirkwood, 1986).

1.2.1 Primary Infection

The progress of the disease generally follows certain characteristic phases. Primary infestations have an initial lag phase that may last 14-21 days and is characterised by low mite numbers and the presence of small focal lesions that are not obvious when looking at the animal. A rapid growth phase follows that is characterised by a sharp increase in mite numbers, lesion size and the presence of circulating antibodies (Bates, 1997a). A decline phase generally follows the rapid growth phase and this is characterised by a decline in both the lesion size and mite numbers. The antibody titre continues to rise at this point, probably due to the continued presence of mite antigen, which stimulates the immune response. Although the mite population is usually completely cleared from the animal in some cases, a residual population of mites is maintained and is able to re-infect the animal once the skin has returned to normal (Bates, 1997a).

Mite-specific antibodies have been detected from mite-infested rabbits (Culbertson, 1935; Fisher, 1983), sheep (Fisher, 1972; Wassall, Kirkwood, Bates et al., 1987) and cattle (Fisher & Wilson, 1977). These appear at the end of the lag phase in sheep (Bates, 1997a). In cattle, IgG was found to increase throughout the

first 7 days of infection and remain at a constant level until over a month post infection (Pruett, Guillot, & Fisher, 1986). A significant increase in mite-specific serum IgG was observed 2 weeks post infestation in sheep, with peak levels occurring at 8-12 week post infestation (van den Broek, Huntley, Machell et al., 2002). Mite-specific serum IgE was found to peak at approx. 13 weeks post infestation and the circulating eosinophils peaked at 11 weeks post infection (van den Broek et al., 2000).

1.2.2 Secondary Infection

The lag phase is extended in a secondary infection. The small lesions characteristic of this phase were observed much earlier than in a primary infestation, with noticeable exudation within 24 hours of infestation and crust formation within 48 hours (Bates, 2000; van den Broek et al., 2000). However, mite numbers remained at a low level and after 42 days post infection the lesions were still small, as seen in the lag phase (Bates, 2000). Similarly, lesions have been found to occur early but progress slowly, in previously infested cattle (Stromberg & Fisher, 1986) and rabbits (Uhlir, 1991) when compared to primary infestations. Mite-specific serum IgE and circulating eosinophils were found to peak much earlier in the infection in previously infected sheep, within 10 days post challenge (van den Broek et al., 2000). In addition, mite-specific serum IgG increased within 1 week post infestation, but then remained at a constant level (van den Broek et al., 2002).

The more severe lesions observed in primary infections may be associated with induction of a host immune response to the mites (Jayawardena, Heller-Haupt, Woodland et al., 1998). The development of mite specific antibodies has been shown to coincide with the onset of dermatitis in cattle (Pruett, Guillot, & Fisher, 1986), which is the product of type 1 hypersensitivity (Stromberg & Fisher, 1986). In addition, immunosuppression of cattle by injection with dexamethasone delayed the production of anti-*P. ovis* antibody and the onset of the scab lesion, providing further evidence for the importance of antibody in the pathogenesis of *P. ovis* infestation (Pruett, Fisher, & DeLoach, 1989).

Several workers have noted a reduction in mite numbers, mite fecundity and lesion development in previously infected animals compared to naïve animals in both

sheep and cattle (Guillot & Stromberg, 1987; Bates, 2000; van den Broek et al., 2000). In cattle, the number of *P. ovis* mites present correlates directly with the extent of the dermatitis and hence the size of the lesion (Stromberg et al., 1986), but this has yet to be tested in sheep. An experiment was conducted where the number of mites found on naïve and previously infected cattle was compared (Stromberg & Fisher, 1986). The authors found that the fecundity of the mites on naïve cattle was high at the beginning of the experiment, but decreased as the mite population grew. In previously infected cattle, mean mite fecundity was found to be lower and remained low throughout the whole experiment (Stromberg & Fisher, 1986). The authors suggested that reduced mite fecundity may be due to ingestion of host immunoglobulin, which subsequently damages or inhibits the digestive processes. Another experiment confirmed these findings but suggested that the reduction in fecundity was due to a reduction in oviposition by the female population rather than an inhibition of egg development (Guillot & Stromberg, 1987). These authors propose that the reduced fecundity is not due to inhibition by the immune response but rather a decrease in the proportion of egg-laying females in the population. These studies did not indicate whether there were any differences in feeding or the development of mites on naïve or previously infected animals. If differences were observed then they may also influence fecundity.

1.2.3 The Lesion – Good for the Mite or Good for the Host?

The question of whether the lesion is advantageous to the mite or the sheep remains controversial. For example, some authors believe that the mite exploits the hypersensitivity response of the host, as it provides a food source (the serous exudate) and a warm and moist microclimate (Stromberg & Fisher, 1986; Bates, 1997a). Bates (1997a) also suggested that the mites cannot survive without the lesion. However, others consider that the scab lesion may be detrimental to the mites. Sinclair & Filan (1991) found that mites taken from the centre of the scab lesion were more likely to have abnormalities, including distorted body shape and round structures in the body cavity, when compared to mites taken from the leading edge of the lesion. The exudate from the lesion may cause the abnormalities observed through the presence of antibody (Sinclair & Filan, 1991) or other

immunomodulators, such as mast cells and eosinophils, which can be found in the exudate (van den Broek et al., 2000). This suggests that the lesion may be used by the sheep to control the mite infection.

1.2.4 Factors affecting the Pathogenesis of Disease

The pathogenesis of sheep scab appears to be dependent on a number of factors such as, age, fleece length, nutritional condition, previous infection, the site of challenge and the virulence of the mites (Bates, 1997b). In addition, the genetic composition of the host may also affect the pathogenesis of the disease. Yearling animals have been found to be affected by more severe scab than older animals, and animals with a “closed” fleece, i.e. lowland breeds, are more affected than are “open” fleeced breeds such as hill sheep. The virulence of the mite also has major effects on the pathogenesis of the disease. There are virulent strains of mite, e.g. the UK Dorset or Cornwall strains that are thought to produce acute disease with severe clinical symptoms, and avirulent strains, such as the UK Welsh or Central Veterinary Laboratory (CVL) strains that produce chronic disease with either no or very few clinical signs (Bates, 1991). For example, Roberts & Meleney (1971) reported that virulent species of mite produced a more persistent infection than avirulent mites. They also observed that virulent strains of mite maintained clinical infection in cattle over the summer months whereas avirulent mites did not. Variation in the efficacy of acaricidal treatments for different mite strains has also been found. Ivermectin at single subcutaneous dose of 200 µg/kg appears to eradicate all mites from a chronic infestation, but leaves residual populations in the acute infection (Bates, 1993). It should be noted, however, that the differences in mite virulence are not static, and the level of virulence of a certain strain can vary between acute and chronic (Bates, 1997b).

1.2.5 Effects of Disease

Sheep scab is considered an important welfare issue because of the intense pain and irritation that the host suffers. There is a correlation between the extent of dermatitis and the number of *P. ovis* mites present (Stromberg et al., 1986), so rapidly expanding mite populations cause rapidly expanding lesions. Kirkwood

rapidly expanding mite populations cause rapidly expanding lesions. Kirkwood (1985) reported cases in sheep where up to 75% of the animal was covered in scab in only eight weeks. As the disease progresses, the pruritis may be so intense that the affected sheep may bite and scratch themselves in an attempt to relieve the discomfort. In some cases, the animals become so over-sensitised that they exhibit a characteristic nibbling response when pressure is applied to the affected areas. Some severely affected animals have been known to undergo a form of epileptic seizure, and this type of seizure has also been observed in experimental sheep scab infestations (Bygrave, Bates, & Daniel, 1993). Weight-loss is another common symptom of the disease. Kirkwood (1980) found that over the 15 week course of the disease the most heavily-infested sheep suffered weight-losses of up to 30% of their weight. Work carried out in cattle suggests that the weight loss may be due to an increased metabolic demand for protein synthesis to compensate for the large loss of serum in the serous exudate throughout the infestation (Meleney & Fisher, 1979; Stromberg et al., 1986). Sheep scab can also have an impact on the birth weight of offspring. Sargison, Scott, Penny et al. (1995) found that lambs born to sheep heavily infested with sheep scab were 10% lighter than lambs born to sheep with only a light infestation. Fleece and pelt quality is also adversely affected. In 1998, it was estimated that poor quality sheep pelts cost the UK leather industry approx. £15 million per year (Coles, 1998). In addition, the carcasses of some animals infested with sheep scab may be considered unfit for human consumption due to the appearance of enlarged lymph nodes (Cameron, 1994; Cochrane, 1994). So *P. ovis* infestation has important economic effects in addition to the welfare issues associated with this disease.

1.3 CONTROL

Control of sheep scab is currently achieved by chemical intervention. Since the re-introduction of sheep scab in 1973, the main chemicals used have been benzene hexachloride, the organophosphates (OPs), the synthetic pyrethroids (SPs) and more recently, the systemic endectocides. Gamma benzene hexachloride (gamma hexachlorocyclohexane (HCH) or lindane) was withdrawn from use in 1984 because of concerns regarding the emergence of resistant mites and consumer

concerns over toxic residues present in lamb (Henderson, 1991). When the disease was re-introduced the government introduced the compulsory national dip, where all flocks were to be treated using a dip containing an “approved” acaricide. Scab approved acaricides had to eliminate a mite infestation in a single treatment and be able to protect the animal against re-infestation for at least three weeks post-treatment (Henderson, 1991). The only scab approved formulations were dips containing either the synthetic pyrethroid, flumethrin (Kirkwood & Bates, 1987), or the organophosphates, diazinon (Kirkwood & Quick, 1981) and propetamphos (Kirkwood & Quick, 1982).

In 1992, sheep scab was deregulated from a notifiable disease, so that chemicals did not have to be approved for use, and the national dip was no longer compulsory. This led to the availability of many different compounds for the treatment and control of sheep scab. For example, the systemic endectocides such as ivermectin, moxidectin and doramectin are now licensed for the treatment of the disease. Unlike doramectin or ivermectin, moxidectin appears to have residual activity that can protect the animal from re-infection for at least two weeks (O'Brien, Brown, Parker et al., 2001). Treatment with ivermectin or doramectin requires a second injection approx. 7 days after the first and transfer of sheep to a clean pasture so that they are protected from re-infestation.

Many different methods have been developed for the application of chemicals for the treatment of scab. Historically, plunge dipping was used, but other methods are now popular, including showers (Kirkwood, Quick, & Page, 1978; Kirkwood, Quick, & Bates, 1983), ivermectin boluses (Bridi, Rehbein, Carvalho et al., 1998; O'Brien, Forbes, Pitt et al., 1999) and pour-ons (Lonneux & Losson, 1992; Losson & Lonneux, 1996). Pour-ons are not effective in the treatment of sheep infested with *P. ovis* (Kirkwood, 1983; Bates, 1993), although they are effective in the treatment of cattle (Losson & Lonneux, 1992).

1.3.1 Mode of action

The organophosphates are nerve toxins that act at synapses and neuromuscular junctions by the inhibition of cholinesterase (Henderson, 1991). The synthetic pyrethroids are also nerve poisons, but they act on axonal transmission by

interfering with sodium channels. As yet, it is not known how these chemicals affect mites, although some mechanisms have been proposed, such as reduced egg laying. The endectocides are thought to induce paralysis in arthropods by interference of glutamate-gated chloride channels (Kane, Hirschberg & Qian, 2000) and affected mites are thought to fall from the host or die due to starvation or dehydration. In addition, there is some evidence to suggest that there is a marked reduction in mite fecundity, numbers of eggs hatching and the pairing of adult males and females on ivermectin-treated cattle, when compared to those on untreated cattle (Guillot & Wright, 1984).

1.3.2 Eradication

Sheep scab has not been eradicated from the UK, despite a government controlled eradication campaign that was in place for almost 20 years and the widespread availability of highly effective chemicals. The eradication campaign is thought to have failed because not every farmer managed to gather all sheep for the annual dip, and some of the dip concentrations used may have been too dilute, and hence not protective (Kirkwood, 1985). In addition effective control is also made more difficult by the mass movement of high numbers of sheep throughout the UK every year, which may allow the disease to spread quickly (Kirkwood, 1985). As a single infected animal can re-infest a whole flock, it is easy to see why even missing a single infested animal can still allow the disease to spread. Theoretically, it should be possible to eradicate sheep scab from the UK; if every sheep in the country was dipped in a “scab approved” formulation within a three week period, then the disease would be eliminated (Kirkwood, 1983). As this has not been successful there is a need to continue to develop and consider novel mechanisms that can effectively control this disease.

1.3.3 Problems with Chemical Control

There are several concerns over the use of chemicals for the control of sheep scab. For example, there are health and environmental concerns over the effects of chemicals, consumer concern over the presence of toxic residues in the meat and fleece, and the emergence of resistant mites to the main chemicals used.

There have been several studies into the effects that the organophosphates, in particular, may have on people regularly exposed to these chemicals. Reports suggest that both acute and chronic illness can result. The symptoms are fairly non-specific and may be confused with influenza (Murray, Wiseman, Dawling et al., 1992), but symptoms such as involuntary muscle spasm leading to severe stomach cramps, sterility in men and abortion in women, and even death due to heart attack have been reported (Bartle, 1991). Also, there are concerns about the effects that the chemicals used in the treatment of scab may affect the environment. OPs are very unstable in soil and are quickly broken down to less toxic products by bacteria (Swanston & Shaw, 1990). However, prior to degradation, they are extremely toxic to wildlife, especially the bird population (Swanston & Shaw, 1990). The use of organophosphate dips has been linked to the pollution of freshwater in Scotland (Littlejohn & Melvin, 1991). This contamination was most abundant during the dipping season, prescribed by the National Dipping Order. Since all flocks had to be treated within a few predetermined weeks, disposal of spent dip was highest at this time.

The endectocide, ivermectin, is thought to be relatively safe in the environment. The avermectins bind strongly to soil, where they are degraded by light and aerobic bacteria (Halley, VandenHeuvel, & Wislocki, 1993). Some studies have suggested that the use of avermectins may lead to increased pasture fouling as some of the insect and bacterial species required for degradation of the faeces are killed by the avermectin residues present in the faeces (Jackson, 1989). However, more recent studies have suggested that ivermectin residues in cow pats do not appear to hinder the degradation of these pats when compared to ivermectin-free pats (Halley, VandenHeuvel, & Wislocki, 1993).

To date, *P. ovis* mites have developed resistance to organophosphates and pyrethroids. Resistance to the organophosphate, diazinon, in South America was reported in 1965 (Henderson, 1991). Pyrethroid-resistant mites were detected in 1995 (Synge, Bates, Clark et al., 1995), and in the UK, mites resistant to propetamphos were found in 1996 (Clark, Stephen, Cawley et al., 1996). As yet, no resistance to the systemic endectocides has been reported in mites, although resistance has been observed in other parasitic organisms, e.g. nematodes.

Ivermectin resistance in the sheep nematode, *Haemonchus contortus*, was first reported in 1985 in South Africa (van Wyk & Malan, 1988; Shoop, 1993).

1.3.4 Alternative control methods

Alternative control measures currently being investigated are the acaricidal properties of plant extracts, and the use of entomopathogenic fungi and genetically modified bacteria.

The use of entomopathogenic fungi has been found to be effective in the control of arthropod pests e.g. ticks (Kaaya, Mwangi, & Ouna, 1996) and so the value of using fungal pathogens to control *P. ovis* infestation is currently being investigated using the fungus, *Metarhizium anisopliae*. This fungus has been shown to be highly pathogenic to *P. ovis* mites (Smith, Wall, & French, 2000). Infection with fungal conidia killed all mites present within 3 days in an *in vitro* experiment, however no *in vivo* trials have been reported.

To date, two different plant extracts, *Lavandula angustifolia* (lavender) and *Artemisia verlotorum*, have been tested for their acaricidal activities. A variety of different extracts of these plants were tested, but it was found that the essential oil extract was the most toxic to the mites (Perrucci, Cioni, Flamini et al., 1994). When the essential oils were tested *in vitro* they resulted in the complete recovery of the lesions, and removal of all mites 7 days after treatment (Perrucci, Flamini, Cioni et al., 2001). They were also found to protect the animal against re-infection for up to one month (Perrucci et al., 2001). This suggests that plant extracts could be useful acaricides in the future.

The bacterium, *Bacillus thuringiensis*, is a potential alternative control option. Recent work suggests that a group of substances produced by *B. thuringiensis*, e.g. thuringiensin, are toxic to larvae of the blowfly, *Lucilia cuprina*, the fly, *Musca domestica* (Pinnock, 1994) and the sheep louse *Bovicola ovis* (Gough, Akhurst, Ellar et al., 2002). These substances are also effective in the control of mite species. The northern fowl mite, *Ornithonyssus sylviarum*, is highly susceptible to thuringiensin, and so this substance may be a valid control method (Pinnock, 1994). To date, no studies have been carried out on the toxicity of *Bacillus thuringiensis* to *P. ovis*.

1.4 VACCINATION

Vaccination is an attractive control solution in many parasitic infections as the problems associated with chemical control, such as residues in meat and fleece and environmental and health concerns (section 1.3.3), would be eliminated and it may offer long-term control. Potential vaccine candidates may be identified by several means, for example, 1) the fractionation of parasite extract and the subsequent selection of protective elements by increasingly refined antigen purification procedures, 2) isolation of components considered essential for parasite survival, 3) the use of antibodies or cells from immune animals to select relevant parasite antigens and 4) the identification of antigens from specific organs that could be targeted by the immune response. The antigens can either be conventional or covert (hidden) antigens. Covert antigens are not normally exposed to the host immune response and may require regular repeat vaccinations in order to maintain the protective effects, as these immune responses will not be boosted by natural infections (Emery, McClure, & Wagland, 1993; Knox, 2000).

Vaccination has been effective in some parasitic species, as exemplified by the successful development of a commercial vaccine against the cattle tick, *Boophilus microplus* (Willadsen, Bird, Cobon et al., 1995). To date, these antigens have been exclusively isolated from the parasite gut. The proteins targeted are not normally presented to the host and therefore do not normally stimulate a response. Because of this, the parasite can be expected to have no defence strategies for dealing with immune responses to these proteins (Munn, 1993). Theoretically, animals immunised with a “hidden antigen” produce antibodies that attach to internal tissues of the parasite, causing damage and associated malfunction. For the *Boophilus* vaccine the protective antigen, Bm86, was found to be a membrane-bound glycoprotein located on the surface of gut cells. Vaccination resulted in fewer ticks engorging, a reduction in the weight of engorging ticks, and reduction in egg laying (Willadsen, Riding, McKenna et al., 1989). Gut antigens have also proven to be effective protective components against the sheep gut nematode, *Haemonchus contortus*, and to date, several protective components have been identified. Contortin, a helical protein that is loosely associated with the luminal surface of the intestinal microvilli of fourth stage larval and adult worms, was found to give

substantial protection against challenge infection with a 78.5% reduction in worm burden (Munn, Greenwood, & Coadwell, 1987). Vaccination of sheep with microgram quantities of H11, a 110kDa microsomal aminopeptidase that is a major component of microvilli of adult *H. contortus* gut, resulted in high levels of protection following a single challenge infection (up to 90% reduction in worm burden; Smith & Munn, 1990). H11-based vaccination was also effective in young lambs and lambs exposed to a trickle challenge infection (Smith & Smith, 1993). Another component identified was termed *Haemonchus* galactose-containing glycoprotein complex, H-gal-GP, a multi-protease glycoprotein from the intestinal membrane of *H. contortus* (Smith, Pettit, Newlands et al., 1999). Vaccination with this component was found to result in an approx. 93% reduction in faecal egg counts and 72% reduction in worm burden (Smith, Smith, & Murray, 1994). Finally, a cysteine protease-enriched antigen extract purified from integral membrane protein extracts of adult *Haemonchus* has also proven to be an efficacious vaccine (Knox, Smith, & Smith, 1999).

Advances have also been made in the development of a vaccine against the sheep blowfly, *Lucilia cuprina*. Four protective antigens have been identified from *L. cuprina* larvae (Bowles, Meeusen, Young et al., 1996; Tellam & Bowles, 1997) using a technique whereby the lymphocytes from repeatedly challenged animals were cultured *in vitro* and antibodies secreted into the culture medium were used to identify larval antigens (Meeusen & Brandon, 1994). Immunisation of sheep with a mixture of these antigens resulted in a significant level of protection from blowfly strike. The larvae on vaccinated animals were observed to be unable to establish a wound and therefore continue their development.

The isolation of components considered essential to parasite survival has resulted in the identification of several parasite proteases as vaccine candidates. As noted above, a cysteine protease-enriched extract has proved useful for vaccination against *Haemonchus*. In addition, immunisation with a water soluble 35 kDa cysteine protease from *H. contortus* gave significant protection to lambs challenged with the same parasite (Boisvenue, Stiff, Tonkinson et al., 1992). Vaccination of sheep and cattle with *in vitro* released cysteine proteases from *Fasciola hepatica* induced high levels of protection (Wijffels, Salvatore, Dosen et al., 1994; Dalton, McGonigle,

Rolph et al., 1996a). Similarly, a cysteine protease inhibitor was shown to block haemoglobin degradation by schistosomes *in vitro*. When tested in mice *in vivo*, the inhibitor was found to reduce worm burdens and decrease fecundity (Wasilewski, Lim, Phillips et al., 1996).

Antioxidant enzymes have also been selected as potential vaccine candidates due to their potentially protective role in neutralising toxic host free radicals. For example, glutathione S-transferase (GST) identified from *Schistosoma mansoni* (Sm28) (Balloul, Grzych, Pierce et al., 1987; Boulanger, Reid, Sturrock et al., 1991) and *S. japonicum* (Sj26) (Liu, Song, Xu et al., 1995a; Liu, Song, Xu et al., 1995b) were shown to have anti-fecundity effects and were host-protective in both mice and pigs. In addition, immunisation with GST isolated from *F. hepatica* resulted in significant protection in sheep (Sexton, Milner, Panaccio et al., 1990) and cattle (Morrison, Colin, Sexton et al., 1996), although the level of protection was not correlated with antibody titre in cattle (Morrison et al., 1996).

To date, limited vaccination studies have been conducted with *P. ovis*. However, it has been shown that animals develop a protective immune response to *P. ovis* infection, and that injection with mite extract can induce similar protective immune responses in both rabbits (Uhlir, 1992) and sheep (Stella, Braun, & Nunez, 1997). Another study, where cattle were immunised with either 100 µg or 10 µg of partially purified mite extracts, showed that animals that received 100 µg immunisations were less susceptible to *P. ovis* challenge and were free of lesions for a greater length of time than the 10 µg immunised animals or the sham-vaccinated controls (Pruett, Temeyer, Fisher et al., 1998). In addition, the vaccinated animals had fewer mites than the untreated controls. This suggests that vaccination could be a feasible control mechanism for treatment of *P. ovis* infestation. Recent studies have shown that partial immunity in sheep can be achieved by vaccination with mite extract and that the protective immunity increases when the mite extract is further fractionated. To date, promising results have been achieved by vaccination with water-soluble and membrane associated fractions of *P. ovis*. Vaccination resulted in a two-fold reduction in mean lesion size and a seven-fold reduction in mite numbers (Smith, Bates, Pettit et al., 2002). Work is currently underway to identify the protective proteins in these fractions. However, to be acceptable to the farmer a

vaccine will probably have to eliminate the lesion completely, because this is the efficacy attained by current chemical control procedures.

Development of a vaccine for *P. ovis* will ultimately depend on the production of recombinant forms of the protective antigen(s). *P. ovis* mites cannot, as yet, be maintained in an *in vitro* environment, and it is not feasible to produce the amounts of protective antigen(s) required for commercial vaccine production using parasite material obtained from donor animals. However, before recombinant molecules can be produced, the antigens present in the mites that may be responsible for the aetiology of the disease, and any protective components, must be identified and characterised.

1.5 ANTIGEN/ALLERGEN CHARACTERISATION

To date, the antigens present in the sheep scab mites remain largely undefined. A number of studies have been conducted to determine the number of antigens present but, as yet, very few have been characterised. These studies have focussed on fractionating the mite extract in SDS-PAGE followed by Western blotting to identify the antigens recognised by immune or infected sheep sera.

A study of antibody responses to *Psoroptes* spp. in bighorn sheep found that sera from infested animals recognised three *Psoroptes* specific bands in a *P. cuniculi* extract, in the range of 12-34 kDa (Boyce, Jessup, & Clark, 1991). Boyce & Brown (1991) identified at least 30 antigens present in *P. ovis* mites using sera from naturally infested bighorn sheep and another study found at least 24 antigens when screening with naturally infested sheep sera (Matthes, Harrison, Shaw et al., 1996). The authors found that 13 of these 24 antigens were recognised by a mouse anti-sheep IgE monoclonal antibody suggesting that these antigens could be associated with the observed allergic response to sheep scab. However, Jayawardena et al. (1998), using mites raised on cattle (they suspected that mites reared on sheep may give non-specific banding on immunoblots when probed with sheep sera), identified six antigenic bands, ranging from 12-183 kDa. A 16 kDa antigen has recently been characterised from *P. ovis* (Pruett, 1999). It is a homologue of the house dust mite group 2 allergen, and has been named, using the same nomenclature as the house dust mite allergens, *Pso o* II (Temeyer, Soileau, & Pruett, 2002). The group 2

allergens are among the major house dust mite allergens; their function remains unknown although recent research suggests that they share some molecular features with male epididymal proteins (Mueller, Benjamin, & Rule, 1998).

The antigens and/or allergens of mites that cause human disease have been extensively characterised and, to date, at least 16 different allergens have been identified. The mite species responsible for human disease include the house dust mites, *Dermatophagoides pteronyssinus*, *D. farinae*, *Euroglyphus maynei* and the storage mites *Lepidoglyphus destructor*. Small antigenic particles released by the mite, probably faecal material, mixes with normal dust and is the cause of allergic disease including asthma, atopic dermatitis and rhinitis, in many humans world-wide. These allergens show marked cross-reactivity between different mite species (Stewart, 1995) and include a wide range of functionally different proteins. For example, many proteases have been shown to be highly allergenic. These allergens include the group 1 allergens, the cysteine proteases (Chua, Stewart, Thomas et al., 1988), as well as the group 3, 6 and 9 allergens, which are serine proteases (Yasueda, Mita, Yui et al., 1986; Stewart, Ward, Simpson et al., 1992; King, Simpson, Moritz et al., 1996). These allergens are recognised by a large proportion of mite-allergic patients and also bind high levels of mite-specific IgE (Stewart, 1995). Other allergens that are not enzymatically active include paramyosin (group 11 allergens), tropomyosin (group 10), fatty acid binding proteins (group 13) and GST (group 8) (Aki, Kodama, Fujikawa et al., 1995; O'Neill, Donovan, & Baldo, 1995; Tsai, Sun, Chao et al., 1999; Puerta, Kennedy, Jiménez et al., 1999). There is a high level of cross-reactivity between house dust mites and *P. ovis* and *P. cuniculi* (Stewart & Fisher, 1986), which suggests that similar allergens may be present in the *Psoroptes* mites. This theory is confirmed by the identification of a group 2 allergen homologue, as mentioned earlier, and a GST homologue from *P. ovis* mites (Lee, Isaac, & Coates, 1999; Temeyer, Soileau, & Pruett, 2002; Lee, Huntley, van den Broek et al., 2002).

Recent work to characterise the allergens present in *P. ovis* has focussed on the fractionation of whole mite extract in SDS-PAGE then probing with a monoclonal mouse anti-sheep IgE antibody to detect proteins that may be involved in the hypersensitivity response. To date, three high molecular weight allergens have

been identified, and N-terminal amino acid sequencing suggests that they are homologous to myosin, paramyosin and vitellogenin (J. Huntley, personal communication). The vitellogenin allergen also has homology to *Der p* 14, an apolipoprotein, which strongly activates the immune response (Epton, Dilworth, Smith et al., 2001) and so may increase the allergenicity of many proteins. In addition, another compound has been isolated by fractionation of whole mite extract, which has cytotoxic effects *in vitro*, and these effects are completely negated by the addition of E64, a cysteine protease inhibitor (J. Huntley, personal communication). This suggests that the compound responsible for cell death *in vitro* is a cysteine protease. However, whether cysteine proteases are responsible for cell death *in vivo* remains to be determined.

1.6 ENZYMATIC CHARACTERISATION

1.6.1 Proteases

Proteases are enzymes that hydrolyse peptide bonds, and these can be further divided into endoproteases that cleave peptide bonds within a polypeptide chain and exoproteases that cleave peptide bonds at either the N or C terminus of the polypeptide chain. There are four main classes of protease: serine, cysteine, aspartyl and metallo-proteases. They are differentiated from each other by the residues that are present at the active site, the substrates on which they act, the types of compound that inhibit this activity, and the conditions under which the activity is optimal (Beynon & Bond, 1989).

Proteases have been implicated in many aspects of parasite survival, such as skin penetration, digestion of food products and evasion of the host immune response (Tort, Brindley, Knox et al., 1999). For example, serine proteases of the blood fluke, *Schistosoma* spp. have been found to aid in the penetration of the cercariae through the skin (Tort et al., 1999). Also, serine proteases released in the excretions/secretions (E/S) of the sheep blowfly larvae, *Lucilia cuprina*, facilitate the establishment of the larvae onto the host skin (Bowles, Carnegie, & Sandeman, 1988; Casu, Eisemann, Vuocolo et al., 1996). These proteases are also involved in digestion of food products, and studies have shown that within 6 hours of hatching all soluble components of the skin have been degraded and ingested by the *L.*

cuprina larvae (Young, Meeusen, & Bowles, 1996). In addition, haemoglobin degradation is due to a combination of cysteine and aspartyl protease activity in *Schistosoma* spp. (Dalton, Smith, Clough et al., 1995; Ghoneim & Klinkert, 1995) and aspartyl proteases in *Haemonchus contortus* (Longbottom, Redmond, Russell et al., 1997). Cysteine proteases have also been isolated from fed but not unfed *Haemaphysalis longicornis* ticks, which may suggest a possible role in digestion (Mulenga, Sugimoto, Ingram et al., 1999; Mulenga, Sugimoto, & Onuma, 1999). Proteases have also been linked with an anticoagulant function e.g. the elastase-like activity of *H. contortus* (Knox & Jones, 1990) and the hookworm, *Ancylostoma caninum* (Hotez & Cerami, 1983), which may help to prolong parasite feeding.

Proteases may also have important roles in development. For example, a combination of serine and metallo-proteases may be important in the egg hatching of *Lucilia cuprina* larvae (Young, Mancuso, Meeusen et al., 2000). In addition, aspartic and cathepsin L-like cysteine proteases are thought to be involved in the development of tick embryos (Fagotto, 1990; Logullo, Vaz Ida, Sorgine et al., 1998).

The cathepsin L cysteine proteases identified from adult *F. hepatica*, are involved in tissue penetration and in nutrient digestion but have also been ascribed other roles. Cathepsin L1 (Smith, Dowd, Heffernan et al., 1993a) may be involved in the evasion of the host immune response, as it has been shown to cleave host immunoglobulin at the hinge region and prevents the attachment of host eosinophils to the surface of the parasites by an antibody-mediated reaction (Smith et al., 1993a; Smith, Carmona, Dowd et al., 1994). Cercarial elastase a serine protease of *Schistosoma* spp. may cleave complement components and host IgG and IgE that bind to the surface of the schistosomulum and therefore may also be implicated in immune evasion (Auriault, Ouaisi, Torpier et al., 1981; Marikovsky, Arnon, & Fishelson, 1988; Marikovsky, Fishelson, & Arnon, 1988; Verwaerde, Auriault, Neyrinck et al., 1988; Marikovsky, Parizade, Arnon et al., 1990; Tort et al., 1999). Other studies suggest that parasite proteases can modulate the host immune response; schistosome serine proteases are capable of regulating the synthesis of specific and non-specific IgE antibodies *in vitro* and *in vivo* in rats (Verwaerde, Auriault, Damonville et al., 1986; Verwaerde et al., 1988). In addition, the major house dust mite allergen, *Der p 1*, is a cysteine protease. The protease function of this allergen

has been found to bias the host response in the direction of a Th2 response by the cleavage of CD25 (Schulz, Sewell, & Shakib, 1998) and increase the levels of IgE synthesised by the cleavage of CD23 (Schulz, Laing, Sewell et al., 1995; Hewitt, Brown, Hart et al., 1995). These findings suggest that the proteolytic activity of the *Der p 1* allergen can create and propagate an allergic environment.

Proteases may also be important in the pathogenesis of the sheep scab mite infestation as they may have roles in lesion development, the hypersensitivity immune response, larval development and digestion. To date, *P. ovis* have been shown to contain a variety of enzymes with phosphatase, esterase, aspartic protease, cysteine protease and aminopeptidase activity being identified (Nisbet & Billingsley, 1999a; 1999b; 2000). It has been suggested that the cysteine proteases of *P. ovis* may share the allergenic properties of the house dust mite (*D. pteronyssinus*) allergen, *Der p 1* (Nisbet & Billingsley, 2000).

1.6.2 Antioxidant Enzymes

Antioxidant enzymes act to limit damage to cells and other components by neutralising the toxicity of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^{\cdot}). ROS are produced by normal metabolism and are also released by host immune cells such as macrophages, eosinophils and neutrophils (Henkle-Dührsen & Kampkötter, 2001). In high concentrations, ROS can damage membrane lipids, proteins and nucleic acids (Petkau, 1986; Southorn & Powis, 1988). In parasitic infections, host-produced oxidants can have severe effects on the survival of parasites *in vitro*, and this is also thought to occur *in vivo*. For example, in *Schistosoma* infection it has been found that a combination of neutrophils, basophils and eosinophils can adhere to opsonized schistosomula and release hydrogen peroxide, which can kill the parasite (Kazura, Fanning, Blumer et al., 1981).

Some antioxidant enzymes studied to date include superoxide dismutase (SOD), catalase, and the peroxiredoxins (Henkle-Dührsen & Kampkötter, 2001). The SOD family is a group of metalloenzymes which catalyse the dismutation of the superoxide anion into hydrogen peroxide and oxygen (LoVerde, 1998). Catalases exist as tetramers of 60 kDa subunits with a prosthetic group of haematin and they

catalyse the reduction of hydrogen peroxide to oxygen and water (Callahan, Crouch, & James, 1988). The peroxiredoxins are the most recent addition to the antioxidant enzyme group. They are a multi-functional enzyme family which catalyses the reduction of hydrogen peroxide to water (Henkle-Dührsen & Kampkötter, 2001). Glutathione S-transferases (GSTs) have also been implicated in an antioxidant role. GST is a multi-functional enzyme which can neutralise toxic compounds produced both by the parasite and external source by conjugating them to glutathione (LoVerde, 1998).

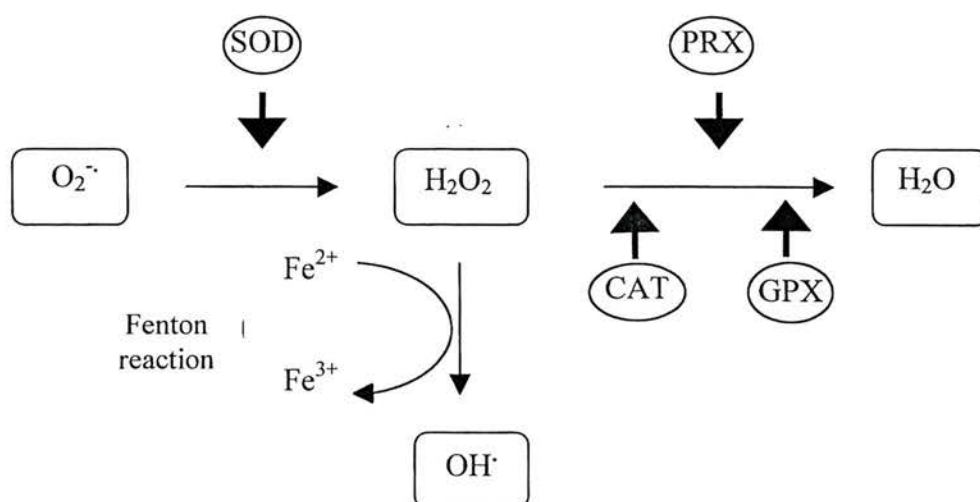


Figure 1.3 The antioxidant enzymes and their role in the neutralisation of the toxic free radicals. SOD, superoxide dismutase; PRX, peroxiredoxin; CAT, catalase; GPX, glutathione peroxidases. This diagram was adapted from Henkle-Dührsen & Kampkötter (2001).

Many authors believe that the parasite antioxidant enzymes are essential for parasite survival, as they deal with both the reactive oxygen species produced by the parasite's own metabolism, and that which is produced by the host in response to infection. For example, work carried out on *Schistosoma* spp. suggests that both SOD and glutathione peroxidase (GPX) may protect the parasite from oxidants released from host immune cells. Both of these antioxidants are located in the tegument of the schistosoma and so may act to suppress the initial chain reaction and prevent the oxidants from entering the parasite. Superoxide anions cannot pass through the tegument, but may still damage it. SOD in the tegument will neutralise the superoxide radical but produce hydrogen peroxide. The hydrogen peroxide can pass

through the tegument, where the GPX enzyme will reduce the hydrogen peroxide to water and oxygen (Figure 1.3, LoVerde, 1998). In addition, the levels and specificity of free-radical scavenging enzymes appear to be related to the level of protection afforded to the parasite. It has been shown in *Schistosoma* spp. that there are higher levels of antioxidants in those adults which are most resistant to attack by the host immune response, compared to susceptible adults (LoVerde, 1998). It has also been shown that two rat intestinal nematode parasites, *Nippostrongylus brasiliensis* and *N. dubius*, have different levels of antioxidant enzymes and these levels have been correlated with the duration of survival in the host. *N. dubius* can persist in the gut for several months and has much higher levels of antioxidant enzymes than *N. brasiliensis*, approximately twice as much SOD activity and 3-4 times as much catalase. *N. brasiliensis*, is expelled from the gut 12-14 days post-infection (Smith & Bryant, 1986; Callahan, Crouch, & James, 1988). To date, only one antioxidant enzyme (GST) has been isolated from *P. ovis* mites (Lee, Isaac, & Coates, 1999; Lee et al., 2002).

1.6.3 Acetylcholinesterase (AChE)

Acetylcholine is a neurotransmitter involved in the passage of nerve impulses over synapses in both the central and peripheral nervous systems. Acetylcholinesterase (AChE) catalyses the hydrolysis of acetylcholine, thereby stopping the passage of the nerve impulses. AChE has been identified from most vertebrates and insects, and is considered to have a housekeeping role, whereby it modulates the amount of acetylcholine present in both neuronal and muscular junctions. If the action of AChE is inhibited, acetylcholine is not removed from the nerve junction and the signal is passed continuously, which may result in the uncontrollable movement of muscles, for example, but ultimately will end in death. AChE is secreted by parasites and may have a number of roles. It may increase cell membrane permeability, serve as anticoagulant and modulate the host immune responses (Pritchard, 1993; Lee, 1996). Furthermore, the presence of AChE in insects is thought to be important in the development of resistance to certain insecticides, e.g. the organophosphates that act by the inhibition of cholinesterase at synapses and neuromuscular junctions (section 1.3.1). Point mutations in the AChE

gene are thought to be responsible for organophosphate resistance in *Drosophila melanogaster* (Fournier, Bride, Hoffman et al., 1992; Mutero, Pralavorio, Bride et al., 1994), although the mechanism for this resistance has not yet been defined.

1.7 AIMS OF PROJECT

In view of the prevalence of sheep scab in the UK and the problems associated with the current chemical control measures, new methods of control are required. Vaccination is a feasible control alternative however, the antigens present in the mites remain largely undefined and potential protective components need to be identified and characterised. The overall aims of this project were to identify *P. ovis* proteins that may be involved in the pathogenesis of the disease, and to identify putative vaccine candidates. The aims were addressed as follows:

1. The proteases present in *P. ovis* were characterised to define the types of proteases present and their ability to degrade substrates that would be found in the parasites natural habitat.
2. A complementary DNA (cDNA) library was prepared from mixed stage *P. ovis* mites for the dual purpose of immunoscreening and expressed sequence tag (EST) analysis. The cDNA library was screened with sera from immunised animals that were subsequently protected from mite challenge to define the proteins that may be involved in the protective immune response.
3. The EST analysis was carried out in an attempt to rapidly define proteins expressed by the mites with relevance to disease pathogenesis.
4. Cysteine proteases identified from the EST analysis were subject to an in-depth sequence analysis to determine the types of cysteine proteases present.

CHAPTER 2: MATERIALS AND METHODS

2.1 PARASITES AND PARASITE EXTRACT

2.1.1 Mites

Psoroptes ovis mites were obtained by Dr. W.D. Smith and his staff from donor sheep maintained at Moredun Research Institute. Briefly, sheep were infected on the withers with approx. 25 ovigerous female mites, and the infection was maintained for 6 – 8 weeks. The donor sheep were then euthanased and sections of skin with mites present were removed with a sharp scalpel and placed in 90 mm Petri dishes. The Petri dishes containing the mites were placed under lamps for 2-3 hours. The mites migrated from the skin section towards the heat of the lamps, and congregated on the lid of the Petri dish. Mites were collected from the lid of the Petri dish using a sharp scalpel dampened with dH₂O. The mites were transferred to a 1.5 ml microfuge tube and maintained on ice. They were washed with 1% (w/v) SDS solution by brief vortexing to remove any sheep material present. The tube was centrifuged briefly and the 1% SDS solution removed by pipette. The mites were then washed 10 times using ice-cold PBS and snap frozen in liquid nitrogen until required.

2.1.2 Mite Extract

Mite extracts were prepared by Mr. D. Pettit; all procedures were carried out on ice or at +4°C, unless otherwise stated. This extract was prepared for use as antigen in vaccination trials. Approximately 8 grams of mites were washed in 40 ml cold homogenising buffer (Phosphate buffered saline (PBS)/1 mM ethylenediaminetetraacetic acid (EDTA)/1 mM phenylmethysulphonyl fluoride (PMSF)) on an end-over-end mixer for 30 minutes. The mixture was centrifuged at 500 x g and the supernatant discarded. Mites were prepared in 40 ml homogenising buffer using both mechanical (Ultra Turrax, IKA), and glass homogenisers. The resulting mixture was transferred to 2 x 25 ml centrifuge tubes and mixed end-over-end for 30 minutes before being centrifuged at 25,000 x g for 30 minutes. The

supernatant was retained and formed the S1 or water soluble mite extract. The pellet was resuspended in 60 ml homogenising buffer + 0.1% Tween-20 and vigorously solubilised using both mechanical and glass homogenisers. Tween-20 is a weak detergent that solubilises proteins that are loosely associated with the membrane. The resulting mixture was transferred to centrifuge tubes, mixed end-over-end for 30 minutes and centrifuged at 25,000 x *g* for a further 30 minutes. The supernatant was retained and formed the S2 or membrane associated extract. The samples were analysed on a 10 % SDS-PAGE (section 2.2.1) and stained using Coomassie Blue. The protein profile of the two fractions was essentially the same. The extracts were stored at -80°C in aliquots until required.

2.2 PROTEIN FRACTIONATION

2.2.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels were prepared following the methods of Laemmli (1970) and cast using Mini Protean[®] II Dual Slab Cell apparatus (BioRad, UK) according to the manufacturer's instructions. Ten percent separating gels were prepared as follows: 4.05 ml dH₂O, 2.5 ml SDS-PAGE separating gel buffer (1.5 M Tris-HCl, pH 8.8), 100 µl 10% (w/v) SDS, 3.30 ml 30% acrylamide/methylene-bis-acrylamide (Severn Biotech Ltd, UK) were mixed and 25 µl TEMED and 75 µl 10% (w/v) ammonium persulphate were added to induce polymerisation. The mixture was poured into the gel apparatus and overlaid with 1 ml dH₂O to allow polymerisation to occur. Following polymerisation, a 4% stacking gel was prepared by combining 6.1 ml dH₂O, 2.5 ml SDS-PAGE stacking gel buffer (0.5 M Tris, pH 6.8), 100 µl 10% (w/v) SDS, 1.3 ml 30% acrylamide/methylene-bis-acrylamide and adding 25 µl TEMED and 75 µl 10% (w/v) ammonium persulphate to induce polymerisation. The stacking gel mixture was overlaid on the separating gel and an appropriate gel comb was inserted to form sample wells on polymerisation. Samples were mixed (v/v) with either reducing or non-reducing sample buffer, loaded into sample wells and electrophoresed at a constant voltage of 200 V for 45 minutes using 1X SDS-PAGE electrophoresis buffer (section 2.2.2). Protein standards (Mark 12™ Invitrogen, UK)

were included on each gel. After electrophoresis, gels were stained with Coomassie Blue (0.25% w/v in 30% methanol, 10% acetic acid v/v) for 30 minutes and destained with Coomassie Blue destain (30% methanol, 10% acetic acid v/v).

2.2.2 SDS-PAGE Electrophoresis Buffer

A 10X stock solution was prepared by dissolving 30 g Tris, 144 g glycine, 10 g SDS in 800 ml dH₂O. The pH was adjusted to 8.3 using 10 M HCl then the solution was made up to 1L with dH₂O.

2.2.3 SDS-PAGE Reducing Sample Buffer

A stock solution of SDS-PAGE reducing sample buffer was prepared by mixing 4.0 ml dH₂O, 1.0 ml SDS-PAGE stacking gel buffer, 0.8 ml glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-β-mercaptoethanol and 0.4 ml 0.05% (w/v) bromophenol blue. Samples were usually mixed 1:1 with the sample buffer and heated to 95°C for 4 minutes prior to electrophoresis.

2.2.4 SDS-PAGE Non-reducing Sample Buffer

SDS-PAGE non-reducing sample buffer was prepared as for reducing sample buffer, except that 2-β-mercaptoethanol was not included and samples were not heated prior to electrophoresis.

2.2.5 Tris Buffered Saline (TBS)

A 10X solution of Tris-buffered saline (TBS) was prepared by combining 20 mM Tris and 150 mM sodium chloride in 800 ml dH₂O. The pH was adjusted to 7.5 using 10 M HCl and the solution was made to 1 litre with dH₂O. The solution was diluted 10-fold before use.

2.2.6 Tris Buffered Saline with Tween 20 (TBST)

TBS (1X) was supplemented with 0.05% Tween 20.

2.2.7 Blocking Buffer

TBS (1X) was supplemented with 5% horse serum (v/v).

2.2.8 Antisera

The antisera used throughout the experiments were obtained from animals that had been vaccinated with either the water-soluble (S1) or membrane associated (S2) extract (section 2.1.2) in trials carried out at Moredun (Smith et al., 2002). Sheep anti-*P. ovis* S1/S2 sera was obtained from animals that were protected from mite challenge as judged by vaccination/challenge trials. A pool of sera was prepared from animals in *P. ovis* Vaccine Trial 3, from 30/Nov/1999. Animal numbers were as follows: CVL SP3747, CVL SP3752, CVL SP3755, CVL SP3756, CVL SP3760, CVL SP3765, CVL SP3767, MRI 1, MRI 2, MRI 3, MRI 4, MRI 5, CVL SP3741, CVL SP3743, CVL SP3750, CVL SP3751, CVL SP3754, CVL SP3759, CVL SP3766, CVL SP 3768, CVL SP3769, MRI6, MRI7, MRI 8, MRI 9 and MRI 10. Antisera were also obtained from animals with natural infestations. Animal numbers were as follows: CVL SP3748, CVL SP3749, CVL SP3758, CVL SP3762, CVL SP3763, CVL SP3764, MRI 11, MRI 12, MRI 13, MRI 14. Negative control sera were obtained from naïve animals. Prior to use in the cDNA library immunoscreen, dot blot experiments were carried out to determine the optimum concentration of the primary antisera, whereby positive plaques were clearly distinguishable from non-recombinant plaques. These experiments also determined if the antisera contained proteins that would cross-react with *E. coli* proteins, and, if so, the antisera were pre-absorbed prior to use (section 2.2.9).

2.2.9 Pre-absorption of Antisera

Occasionally antisera were found to cross-react with *E. coli* or phage proteins resulting in a high level of background staining and the increased possibility of misinterpretation as false positives. To remove the cross-reacting antibodies, the sera were incubated with an *E. coli* phage lysate (Stratagene, UK). Briefly, the *E. coli* phage lysate was diluted 1:10 in TBST and bound to four 82 mm nitrocellulose discs (Hybond C-extra, Amersham) by incubation at room temperature for 30 minutes. The membranes were washed in TBST, after which non-specific protein sites were blocked by incubation in blocking buffer for 1 hour. After extensive washing in TBST, 100 µl of antisera was diluted in 5 ml TBST. One nitrocellulose membrane was incubated in the diluted antisera at 37°C for 10 minutes to remove any cross-

reacting antibodies. This process was repeated until all four membranes had been used. The pre-absorbed antiserum was collected and diluted optimally in blocking buffer and stored at +4°C until required.

2.2.10 Procedure for Western Blot

Proteins fractionated by SDS-PAGE can be transferred to nitrocellulose membranes for identification of specific proteins by their reaction with antisera (Towbin, Staehelin, & Gordon, 1979). This procedure was conducted using a semi-dry Western Blotting system, MilliBlot™ Graphite Electroblotter 1 (Cat. No. MBBDGE001, Millipore, UK) according to the manufacturer's instructions. Three sheets of filter paper (Whatman 3 MM Chr) were cut to approx. 17 cm x 17 cm. The first sheet was soaked in anode buffer A (18.72 g of Tris in 500 ml dH₂O) and placed over the anode. The second sheet was soaked in anode buffer B (1.51 g Tris in 500 ml dH₂O) and placed directly over the first sheet. An Immobilon-P membrane (Millipore, UK) was cut to the required size (approx. 7cm x 9cm), soaked in methanol and washed in anode buffer B before being placed on top of the upper sheet of filter paper. The gel was placed on the Immobilon-P membrane and covered with the final sheet of filter paper, soaked in cathode buffer (1.51 g Tris and 0.25 g glycine in 500 ml dH₂O). The cathode was then placed onto the electroblotter and transfer was allowed to proceed for 45 minutes at 150 V per gel. The Immobilon-P membrane was stained with 0.1% Ponceau stain (Sigma, UK) in 5% acetic acid to visualise the transferred proteins. The protein standard lane was first removed from the rest of the blot and stained in 0.25% Coomassie Blue. Non-specific protein binding was prevented by incubating the membrane in blocking buffer overnight at room temperature with constant agitation. The blots were washed 3 x 10 minutes in TBST, incubated in primary antibody (generally sheep anti-*P. ovis* S1/S2) for 1 hour at room temperature with constant agitation and then washed extensively in TBST. The membranes were incubated in secondary antibody (Horse Radish Peroxidase donkey anti-sheep IgG, at 1:500 dilution, Scottish Antibody Production Unit (SAPU)) for 1 hour. The blots were stained using Sigma Fast 3,3'-diaminobenzidine (DAB) tablets, following the manufacturer's instructions.

2.3 PROTEASE CHARACTERISATION

2.3.1 Assay buffers

For protease analyses, 0.1 M or 0.2 M assay buffers were prepared for the following pH ranges: pH 3 to 6, acetate buffer; pH 7 to 9, phosphate buffer; pH 10 to 11 glycine sodium hydroxide buffer according to the methods of Henry, Connan, & Winkman (1974).

2.3.2 Purification of IgG

IgG was purified from sheep sera using Protein G-Agarose (Boehringer Mannheim) according to the manufacturer's instructions. Protein G is a bacterial cell wall constituent that has specific binding affinity for the Fc portions of immunoglobulins, specifically most subclasses of IgG. Protein G was chosen as it has a higher binding capacity for sheep IgG than Protein A. Sheep sera were bound to the protein G agarose in a microfuge tube for 20 minutes at room temperature with constant agitation. After centrifugation at 13,000 x g for 40 seconds, the supernatant was discarded and the sample was washed in buffer comprising 20 mM sodium phosphate, 150 mM sodium chloride, 2 mM EDTA at pH 7.0, centrifuged briefly, and the supernatant discarded. This process was repeated 10 times. The purified IgG was eluted using a buffer comprising 100 mM glycine at pH 2.7 and centrifugation at 13,000 x g for 40 seconds. The supernatant containing the purified IgG was collected into a neutralisation buffer comprising 1.0 M Tris, pH 9.0. The absorbance at 280 nm was recorded for each purified sample to give an indication of the protein concentration; according to the manufacturer's protocol, one A_{280} unit is equivalent to approx. 0.8 mg/ml IgG. Finally, 10 μ l of each sample was analysed by 10% SDS-PAGE under reducing conditions. The purified IgG was stored at + 4°C until required.

2.3.3 Substrate Gel Analysis

Proteolytic enzymes can be visualised and their molecular weight estimated using substrate gels. A protein substrate is co-polymerised with the gel matrix. After electrophoresis of non-reduced protein samples, gels are incubated overnight at

the appropriate pH and temperature and zones of proteolysis visualised by Coomassie Blue counterstaining. The water-soluble (S1) and membrane-associated (S2) extracts (approx. 10 µg protein, section 2.1.2) were fractionated using 10% substrate SDS-PAGE. The protein substrates used were gelatin, haemoglobin, azocasein, and fibrinogen (Sigma, UK). Gels were prepared as described for SDS-PAGE (section 2.2.1) except that 10 mg of the protein substrate was dissolved in the separating gel mix. After electrophoresis at 200 V for 45 minutes with one change of ice-cold electrophoresis buffer to preserve enzyme activity, gels were washed for one hour in four changes (100 ml each) of 2.5% Triton X-100 to remove the SDS. The gels were then incubated in assay buffers (0.1 M, section 2.3.1) with or without the addition of 2 mM dithiothreitol (DTT), for 16 hours at 37°C. DTT is a reducing agent which activates cysteine proteases and, as cysteine proteases are generally active at acidic pH, the DTT was added to the pH 3 – 5 buffers when required. Zones of proteolysis were visualised by Coomassie Blue staining followed by standard destaining for 15 minutes, as described earlier (section 2.2.1). After this, destaining was continued using 10% (v/v) acetic acid, changed every 30 minutes until clear zones of proteolysis were visible on a dark blue background.

2.3.4 Degradation Assays

Solutions of fibrinogen (Fraction 1 from Sheep Plasma) and haemoglobin (Bovine) (2 mg/ml) and of IgG and fibronectin (Bovine) (1 mg/ml) (all from Sigma) were prepared in PBS. Ten microlitres of individual substrates were mixed with 3.5 µl (4 µg) of either S1 or S2 extracts (section 2.1.2) or with 3 µl of PBS to act as a substrate only control. Two microlitres of assay buffer (0.2 M) (section 2.3.1) were added and the samples incubated for 16 hours at 37°C. Samples incubated with acidic buffers were neutralised before electrophoresis by the addition of an equal volume of 1 M Tris-HCl, pH 9.0. Electrophoresis was carried out under reducing conditions at 200 V for 45 minutes. The gel was then stained with Coomassie Blue as described earlier (section 2.2.1).

Degradation of azocoll, elastin-orcein and keratin-azure (all from Sigma, UK) by mite extracts was analysed using a spectrophotometric assay. Briefly, the protein substrate (1 mg) was mixed with 100 µl of assay buffer (0.2 M) containing 50 µg/ml

ampicillin (pH 3-5 contained 2 mM DTT) and 1 μ l (1.5 μ g) of the S1 or S2 extract added. Samples were incubated at 37°C for 16 hours and centrifuged briefly at 13,000 x g. The absorbance of the supernatant was then determined spectrophotometrically at 520 nm (azocoll), 550 nm (elastin-orcein) or 620 nm (keratin-azure) using a Beckman DU[®]-650 spectrophotometer.

2.3.5 Inhibitor Studies

Class-indicative protease inhibitors were either added to the overnight incubation buffer for the substrate gels, or included in the degradation assays. The inhibitors (Sigma, UK) used and their final concentrations were: 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF, 2 mM); L-trans-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane (E64, 10 μ M); Pepstatin A (10 μ M) and 1,10 phenanthroline (1 mM). These inhibitors were chosen as they inhibit each of the four main protease classes, i.e. serine, cysteine, aspartyl and metallo-proteases, respectively.

2.3.6 Antibody Inhibition Studies

IgG was purified (section 2.3.2) from: 1) infection sera, 2) naïve control sera or 3) sera from animals immunised with S1 or S2. To evaluate inhibition of protease activity, 3.5 μ l S1 or 7.5 μ l S2 extract (10 μ g, section 2.1.2) was mixed with 1 μ l dH₂O to act as a control or 1.4 μ l (1 μ g), 7 μ l (5 μ g) or 14 μ l (10 μ g) of the purified IgG (from section 2.3.2) and incubated at 37°C for one hour prior to electrophoresis in 5% native gels containing 0.1% gelatin. Native gels were prepared as for standard SDS-PAGE except all SDS was omitted from the gel mixture and all buffers including the sample buffer, so that the samples were analysed under completely non-reducing conditions. The gels were incubated overnight at 37°C in 0.1 M acetate buffer (pH5) with or without the addition of 2 mM DTT and stained with Coomassie Blue as described in section 2.3.3.

2.3.7 Esterase and Acetylcholinesterase (AChE) Assay

Ten micrograms of the S1 and S2 extract (section 2.1.2) were fractionated in a 7.5% native gel as described above. The gel was stained for the presence of esterases following the method of Grunder, Sartore, & Stormont (1965). Briefly,

gels were incubated in 20 ml PBS containing 5 mg/ml Fast Blue RR salt (Sigma, UK) and 1 ml 2% naphthyl acetate/acetone at room temperature until bands of enzyme activity were identified. A second gel was stained for the presence of AChE using the method of Karnovsky & Roots (1964). The gels were placed in a solution comprising 26 ml 0.77mg/ml acetylthiocholine iodide/0.1 M phosphate buffer (pH 7), to which 2 ml 0.1 M sodium citrate, 4 ml 30 mM copper sulphate, 4 ml distilled water and 4 ml 5 mM potassium ferricyanide were added. The gel was incubated at 37°C on a rocking platform until bands were visible.

A more sensitive spectrophotometric assay was employed for the analysis of acetylcholinesterase activity in mite extracts (Ellman, Courtney, Andrew et al., 1961). Acetylthiocholine iodide acts as substrate, which in the presence of acetylcholinesterase, is hydrolysed to produce thiocholine and acetate. Thiocholine then reacts with dithio-bis-nitrobenzoate (DTNB) to produce a yellow colour, the intensity of which is related to the amount of acetylcholinesterase activity. Briefly, the assay solution consisted of 5 µl of acetylthiocholine iodide (21.67 mg/ml, Sigma, UK in 0.1 M phosphate buffer, pH 8.0) and 25 µl of 5'5'-dithiobis-2-nitrobenzoic acid (DTNB, 39.6 mg/ml, Sigma UK)/sodium biocarbonate (15 mg/ml in 0.1 M phosphate buffer, pH 7.0) in 750µl PBS. As a positive control, 12.5 µl of acetylcholinesterase (Sigma, UK) at a concentration of either 500 units/ml or 0.5 units/ml was added to the assay solution, and the change in absorbance at 410 nm at room temperature was monitored using a Beckman DU[®]-650 spectrophotometer. To determine the acetylcholinesterase activity in the mite extract, either the S1 or S2 extract (12.5 µl, section 2.1.2) was added to fresh assay solution and the change in absorbance at 410 nm was observed as above.

2.4 GENERAL MOLECULAR BIOLOGY PROCEDURES AND SOLUTIONS

All solutions were prepared using distilled water unless otherwise stated. When required, solutions were sterilised before use by autoclaving at 121°C for 15 minutes.



2.4.1 Agarose Gel Electrophoresis

Tris-Acetic acid-EDTA Buffer (TAE)

This was prepared as a 50X solution, consisting of 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA in 800 ml dH₂O. The pH was adjusted to 8.0 using 10 M HCl and the solution made up to 1L with dH₂O.

Agarose Gel Sample Loading Buffer

Agarose gel sample buffer was prepared by dissolving 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in dH₂O. Sample DNA was mixed with the loading buffer (5:1 v/v) prior to electrophoresis.

Agarose Gel Electrophoresis

Generally, 0.8% agarose gels were used. Agarose (0.8 g) was dissolved in 100 ml 1X TAE by heating in a microwave oven at full power (950 W) for two minutes. Once cooled to approx. 45°C, 5 µl 10 mM ethidium bromide was added and the solution was swirled to mix. The solution was then poured into a gel template (Pharmacia GNA-100 minigel apparatus), an appropriate gel comb was inserted and the gel was allowed to set. The gel was placed in the electrophoresis tank and submerged in 1X TAE. Samples were mixed with agarose gel sample loading buffer prior to loading into the sample wells. DNA size markers (0.07-12.2 kb, Roche, UK) were included in each gel to provide approximate DNA sizes. The gels were run at 80 V for 40 minutes. The DNA was visualised under UV light.

2.4.2 Polyacrylamide Gel Electrophoresis (PAGE)

7.5% PAGE Gel

A 7.5% gel was prepared by combining: 2 ml 5X Loening buffer (see below), 2.5 ml acrylamide/methylene-bis-acrylamide (Severn Biotech Ltd, UK), 5.5 ml distilled water, 100 µl 10% ammonium persulphate and 25 µl TEMED. The gel was poured into the standard BioRad Mini-Gel System, and a comb inserted to form sample wells.

Loening Buffer

This was prepared as a 5X aqueous solution comprising 36 mM Tris base, 30 mM sodium dihydrogen phosphate and 1 mM Na₂EDTA.

Silver Staining

This method of staining is highly sensitive and can detect much lower quantities of DNA than standard agarose gels. Following electrophoresis, polyacrylamide gels were fixed for 15 minutes in a solution comprising 10% (v/v) ethanol and 0.5% (v/v) acetic acid in dH₂O. The gels were stained for 30 minutes in 11.2 mM silver nitrate and rinsed twice briefly in distilled water. The staining was visualised with developing solution (750 mM sodium hydroxide and 0.1% (v/v) formaldehyde in dH₂O). Once the gels had stained sufficiently, the reaction was stopped using excess 70 mM sodium carbonate.

2.4.3 Luria-Bertani (LB) Broth

The following chemicals were combined: 10 g sodium chloride, 10 g tryptone, 5 g yeast extract and made up to 1 litre with dH₂O. The pH was adjusted to pH 7.5 with 5 M sodium hydroxide and the broth sterilised by autoclaving.

2.4.4 LB Agar

Agar (20 g) (Bacto™ Agar, Becton Dickinson) was added to 1 litre of LB broth and the media sterilised before use.

2.4.5 LB Top Agarose

Agarose (0.7% w/v) was added to 1 litre of LB broth and the media sterilised prior to use.

2.4.6 Antibiotic Stock Solutions

Tetracycline

A 1000X stock solution of tetracycline (Sigma, UK) was prepared by dissolving 15 mg tetracycline in 1 ml dH₂O. The stock solution was stored at -20°C in the dark.

Kanamycin

A 500X stock solution of kanamycin (Sigma, UK) was prepared by dissolving 25 mg kanamycin in 1 ml dH₂O. The stock solution was stored at -20°C.

Chloramphenicol

A stock solution of chloramphenicol (Sigma, UK) was prepared by dissolving 34 mg chloramphenicol in 1 ml 100% ethanol and the stock solution was stored at -20°C until required.

Ampicillin

A 500X stock solution of ampicillin (Stratagene, UK) was prepared by dissolving 25 mg of ampicillin in 1 ml dH₂O. The stock solution was then stored at -20°C.

2.4.7 LB/tet Plates

LB agar (500 ml) was melted in a microwave oven, cooled to approx. 50°C and 500 µl tetracycline stock solution (final concentration of 15 µg/ml) was added. Plates (90 mm) were poured and stored at +4°C until required.

2.4.8 LB/kan/cam Plates

LB agar (500 ml) was melted in a microwave oven, cooled to approx. 50°C, 1 ml kanamycin stock solution (final concentration of 50 µg/ml) and 2 ml chloramphenicol (final concentration of 150 µg/ml) was added. Plates (90 mm) were poured and stored at +4°C until required.

2.4.9 LB/amp Plates

LB agar (500 ml) was melted in a microwave oven, cooled to approx. 50°C and 1 ml ampicillin stock solution (final concentration of 50 µg/ml) was added. Plates (90 mm) were poured and stored at +4°C until required.

2.4.10 SM Buffer

An aqueous solution which comprised 0.1 M sodium chloride, 1 mM magnesium sulphate, 50 mM Tris and 0.01% gelatin (w/v) was prepared, adjusted to pH 7.5 with HCl and made up to a final volume of 1 litre with dH₂O.

2.4.11 XL1 Blue *E. coli* Cells

XL1-Blue host cells were obtained from Clontech Laboratories (UK). A scraping of frozen cells was streaked onto LB/tet plates and incubated overnight at 37°C.

2.4.12 Liquid Culture of XL1-Blue Cells

Fifty ml of LB broth were supplemented with magnesium sulphate and maltose to final concentrations of 10 mM and 140 mM respectively. One colony of XL1-Blue *E. coli* as described in section 2.4.11 was inoculated into this medium and the culture allowed to grow for 4-6 hours at 37°C or overnight at 30°C with constant shaking at 225 rpm. The culture was centrifuged at 3,000 x g for 10 minutes at room temperature and the supernatant was discarded. The pelleted cells were then resuspended in sterile 10 mM magnesium sulphate at a density equivalent to an OD₆₀₀ of 0.5.

2.4.13 Bacterial Liquid Cultures

The colony of interest was inoculated into 10 ml LB broth supplemented with required antibiotics at appropriate concentration and incubated overnight at 37°C with shaking at 225 rpm.

2.4.14 Purification of DNA from *E. coli* Liquid Cultures

Plasmid DNA was purified from bacterial liquid cultures using the Promega Wizard® Plus SV Minipreps DNA Purification System. This kit is an adaptation of the alkaline lysis method of DNA purification first described by Birnboim & Doly (1979). A 10 ml bacterial culture (section 2.4.13) was centrifuged at 3,000 x g for 10 minutes to pellet the bacterial cells. The pellet was resuspended in 250 µl of cell resuspension buffer. The cells were lysed to release the plasmid DNA, the reaction

neutralised and the cell debris (comprising precipitated protein and bacterial genomic DNA) removed by centrifugation at 13,000 x g for 10 minutes. The supernatant was transferred to a Wizard® Plus SV Miniprep Spin Column, centrifuged at 13,000 x g for 1 minute and the flow-through discarded. The Spin Column membrane was washed twice in wash buffer containing ethanol to remove any contaminants and the bound DNA eluted using 50 µl nuclease-free water. An aliquot of the purified DNA was removed for agarose gel analysis and spectrophotometric analysis at 260 and 280 nm using a Beckman DU-600 spectrophotometer. The remainder of the sample was stored at -20°C.

2.4.15 Purification of DNA from PCR Reactions

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, catalogue no. 28104) following the manufacturers instructions. The DNA was bound to the QIAquick silica-gel membrane in the QIAquick column in the presence of chaotropic salts. This ensured that only the DNA bound to the column, while the contaminants passed through the membrane. The chaotropic salts were removed from the membrane by an ethanol wash followed by centrifugation at 13,000 x g for 1 minute. To ensure that no residual ethanol was present that might interfere with subsequent reactions, an additional centrifugation was carried out at 13,000 x g for 1 minute. The DNA was eluted in 50 µl dH₂O and an aliquot removed for spectrophotometric and agarose gel analysis. The remaining DNA was stored at -20°C until required.

2.4.16 Purification of DNA from TAE Agarose Gels

A procedure similar to that described in section 2.4.15 was followed, using the QIAquick Gel Extraction Kit (Qiagen, catalogue no. 28704). The gel was viewed under UV illumination using a Crosslinker Transilluminator (Hybaid) and the DNA band of interest was excised using a clean, sharp scalpel. The gel slice was solubilised in a buffer containing chaotropic salts and pH indicators by incubation at 50°C for 5 minutes with occasional vortexing. This buffer was used as it ensured optimum binding to the silica-gel membrane. The solubilised gel was then added to

the silica-gel membrane and the DNA allowed to bind, after which the protocol for section 2.4.15 was followed.

2.5 COMPLEMENTARY DNA (cDNA) LIBRARY SYNTHESIS AND IMMUNOSCREENING

2.5.1 Messenger RNA (mRNA) Extraction

Messenger RNA was prepared from *P. ovis* mites (section 2.1.1) using an mRNA extraction kit (Stratagene, U.K.). Approximately 700 mg of snap-frozen mites were added to 5 ml mRNA denaturing solution in a mortar and pestle, which was pre-cooled to -80°C . The resultant mixture was powdered and allowed to thaw to room temperature before the addition of 10 ml elution buffer and gentle mixing. The sample was then dispensed in 1.5 ml aliquots into 15 sterile eppendorfs and centrifuged at $12,000 \times g$ to remove any protein. The supernatants were pooled, applied to oligo dT cellulose (0.4 g/ml) and incubated at room temperature for 15 minutes with constant agitation. After centrifugation at $700 \times g$ for 3 minutes at room temperature, the supernatant was discarded and the oligo dT cellulose was washed in three changes of high salt buffer, followed by two changes of low salt buffer. The mRNA was eluted from the oligo dT cellulose by the use of a push-column with elution buffer at 68°C . The mRNA was ethanol-precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2 and 3 volumes of 100% ethanol. The samples were then incubated overnight at -20°C and centrifuged at $14,000 \times g$ for 30 minutes at $+4^{\circ}\text{C}$, after which the supernatant was removed and the pellets dried under vacuum for 2-5 minutes. The mRNA pellet was then dissolved in 40 μl elution buffer and the concentration of mRNA present was measured by spectrophotometric analysis at 260 nm and 280 nm. According to the manufacturer's protocol, one A_{260} unit is equal to 0.04 $\mu\text{g}/\mu\text{l}$ mRNA. The mRNA quality was also assessed by 0.8% agarose gel electrophoresis and for analysis at higher sensitivity, the mRNA was also fractionated on 7.5% PAGE and visualised by silver-staining (section 2.4.2).

2.5.2 Library Synthesis

A cDNA library was generated from mRNA from mixed-stage *P. ovis* mites (prepared as in sections 2.1.1 and 2.5.1) using the SMART™ cDNA Construction Kit (cat: K1051-1 Clontech Laboratories, UK). This kit utilises SMART (switching mechanism at 5' end of RNA transcript) technology and a type of Moloney murine leukaemia virus (MMLV) reverse transcriptase (RNase H point mutant, Superscript II, Gibco RBL, UK) with terminal transferase activity that adds 3-5 residues, generally dC's, to the end of the first strand cDNA. The technology then uses a SMART III™ oligonucleotide with a poly G tail, which binds to the dC region of the first strand cDNA and extends the template for reverse transcriptase (see Figure 2.5). The reverse transcriptase then switches templates to the SMART III™ oligonucleotide and a complete copy of the original mRNA template is generated with the SMART oligonucleotide at the 5' end. A greater number of full length sequences are generated from this method of library construction, as the efficiency of the reverse transcriptase in adding residues is greatest when the enzyme has reached the end of the RNA transcript. Therefore, the SMART oligonucleotide is generally only added to full-length sequences.

The cDNA library was constructed following the manufacturer's instructions. The first strand was synthesised using the long-distance PCR method. Briefly, the first strand synthesis reaction was primed with a modified oligo (dT) primer, using reverse transcriptase. The second strand was synthesised by PCR and utilised the 3' PCR primer CDS III and the SMART III oligonucleotide. The DNA polymerase activity from the PCR reaction was inactivated by proteinase K digestion and the double stranded cDNA was prepared for ligation into the λ TriplEx2 vector by digestion with the endonuclease *Sfi* I and size fractionation. Size fractionation removes any unused nucleotides, excess *Sfi* I and small cDNA. Three parallel ligation reactions were set up to ensure that the library obtained from the cDNA was of the best quality possible. Each ligation reaction had a different ratio of cDNA to vector. The ligation reactions were incubated at 16°C for 16 hours and then packaged using the Gigapack III Gold packaging extract (Stratagene, UK). This system was chosen as it had a high transformation efficiency of 1×10^9 plaque forming units (pfu)/ μ g DNA.

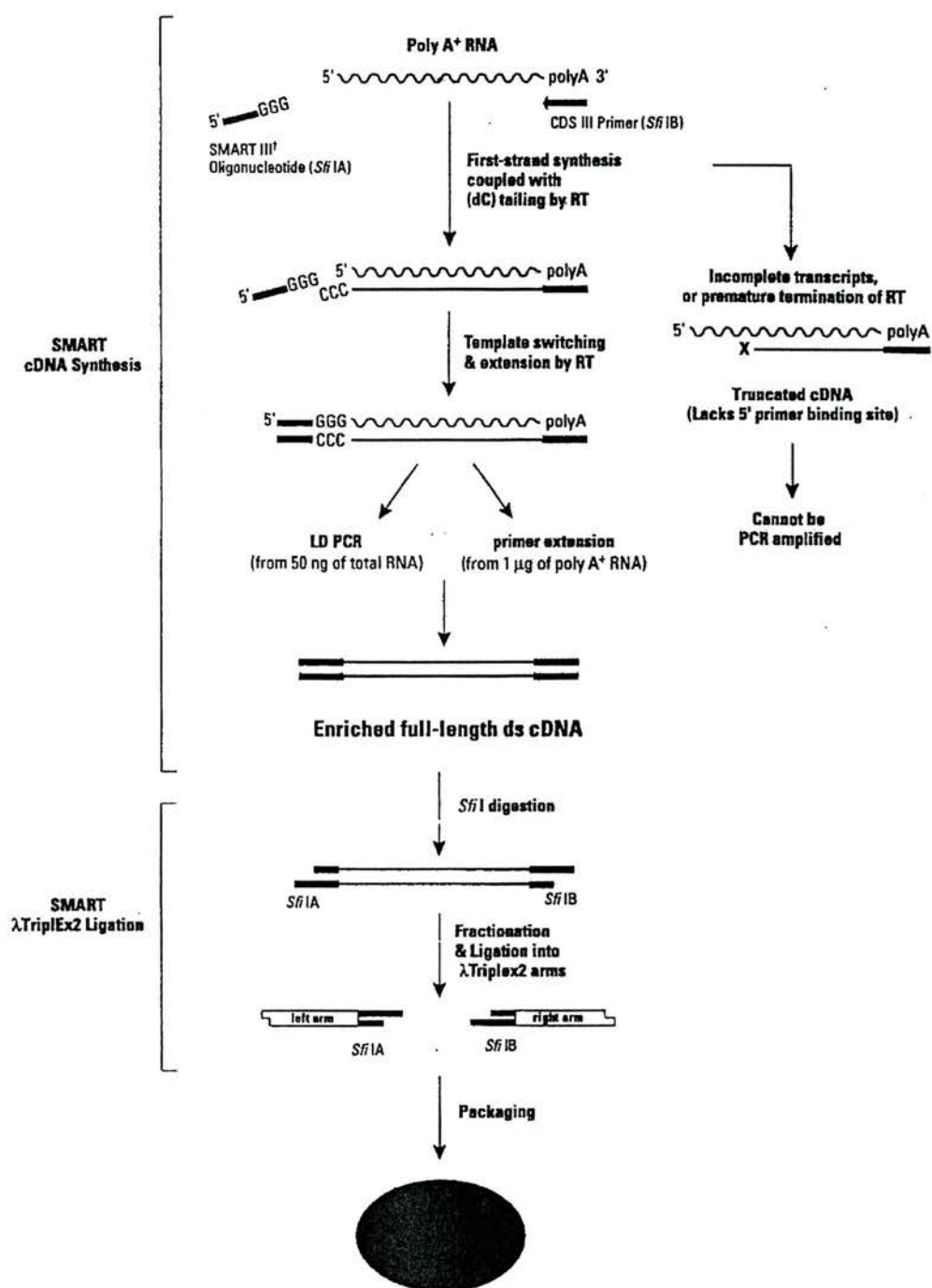


Figure 2.5. A flow chart of the protocol for the SMART™ cDNA Synthesis Kit. This diagram was adapted from the Clontech SMART™ cDNA Library Construction Kit, version # PR92334

2.5.3 Titre and Percentage Recombinants of the Primary cDNA Library

The titre of the library was determined by plating serial dilutions of the packaged primary cDNA library reaction. Ten μl of the appropriate dilution of the primary cDNA library was added to 200 μl of XL1 Blue cells (prepared as section 2.4.12). The phage particles were allowed to adsorb for 15 minutes at 37°C without shaking. LB top agarose (3 ml), melted and cooled to 48°C, was added to the host cell/phage mixture and immediately poured onto pre-warmed 90 mm LB agar plates. The plates were then inverted and incubated at 37°C for 16 hours.

The ratio of recombinant: non-recombinant plaques was determined using blue/white colour selection. This is possible due to the location of the TriplEx2 vector polycloning site within the LacZ⁻ gene, which encodes β -galactosidase. Those colonies without a cDNA insert produce an intense blue colour in the presence of the 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) substrate as the LacZ⁻ gene remains intact. The colonies that contain cDNA inserts do not produce the blue colour as the presence of the cDNA insert disrupts the LacZ⁻ gene. Thus non-recombinant plaques were blue whilst recombinant plaques were white. For blue/white colour selection, 15 μl of an aqueous solution of 0.5 M isopropyl-1-thio- β -D-galactopyranoside (IPTG) and 50 μl of 250mg/ml X-gal in dimethylformamide must be added to the top agarose when plating out the cDNA library.

2.5.4 Preparation of Phage Stock

Recombinant plaques of interest were picked and placed into 500 μl SM buffer and 20 μl chloroform. They were vortexed to release the phage particles and incubated at room temperature for 1-2 hours, or for 2 days at +4°C, to allow the phage to diffuse into the supernatant.

2.5.5 Analysis of Insert Size

Twenty recombinant colonies and four non-recombinant colonies were chosen at random from the library titre plates and used to prepare phage stocks. A freeze/thaw template of each plaque was made by mixing 20 μl of the phage stock

with 20 µl dH₂O. This mixture was subjected to 3 cycles of freezing at -80°C for 5 minutes and thawing at +70°C for 5 minutes. A PCR reaction was carried out with the freeze/thaw template mixture to determine the size of inserts present, using the 5' and 3' sequencing primers supplied with the cDNA construction kit (Clontech). The primer sequences were as follows:

5' sequencing primer: 5'-TCC GAG ATC TGG ACG AGC-3'

3' sequencing primer: 5'- TAA TAC GAC TCA CTA TAG GG-3'.

All reagents for the PCR reaction were purchased from Bioline, UK. The PCR reaction consisted of 2.5 µl 10 X PCR buffer, 5.0 µl dNTP's, 2.5 µl 10 µM 3' sequencing primer, 2.5 µl 10 µM 5' sequencing primer, 0.75 µl 50 mM MgCl₂, 1 µl DNA *Taq* polymerase, 8.25 µl sterile dH₂O and 2.5 µl target DNA. The reaction conditions were as follows: 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes and a final extension of 72°C for 7 minutes. The PCR products were analysed by electrophoresis in 0.8% agarose gel and visualised under UV light using ethidium bromide.

2.5.6 Library Amplification

The primary library was amplified to ensure that it was stabilised for long-term storage. After each round of immunoscreening and selection of positive plaques, the remaining plaques were eluted from the plate. Briefly, 10 ml SM buffer were added to each plate and these were then incubated at +4°C overnight with gentle shaking. The resulting λ-phage lysate was poured into a 50 ml conical tube, then each plate was washed with a further 2 ml SM buffer and the washings were added to the pooled lysate. Chloroform was added to a final concentration of 5% (v/v) and mixed. The pooled lysate was then incubated at room temperature for 15 minutes and cell debris was removed by centrifugation at 500 x g for 10 minutes. The supernatant was removed and constituted the amplified library. Chloroform was added to a final concentration of 0.3% (v/v) to retard any bacterial growth and the amplified library was stored at +4°C. The titre of the amplified library was obtained as described in section 2.5.3.

2.5.7 Primary Antibody Used for Immunoscreening

Antibody for immunoscreening was derived from a pool of sera collected from animals that had been vaccinated with either the water-soluble (S1) or membrane associated (S2) mite extract in a trial carried out at Moredun Research Institute (Smith et al., 2002). Sera were collected from animals that were found to have some protection against mite infection in vaccination/challenge experiments. Serum was pooled from 12 animals that had been vaccinated with the S2 extract and 14 animals that had been vaccinated with the S1 extract.

2.5.8 Primary Antiserum Definition prior to Immunoscreening

Prior to use, the sera were tested on immunoblots to define the protein recognition profile. Briefly, 10 µg of the S1 and S2 extract (section 2.1.2) were fractionated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoreactive proteins were identified using the procedure described in section 2.2.10. Pooled sera were used at a working dilution of 1:500.

In addition, an experiment was conducted to determine the optimum concentration of the primary antibody for use in library immunoscreening such that plaques recognised by the antisera would be clearly distinguishable from non-recombinant plaques.

2.5.9 Immunoscreening

The cDNA library was plated on a lawn of XL1 Blue cells at a density of 3,000 pfu/150 mm plate and incubated at 42°C for 3-4 hours until plaques were just visible. After being overlaid with Hybond C-extra nitrocellulose membranes (Amersham) impregnated with 10 mM IPTG (Promega, UK), the plates were inverted and incubated at 37°C for 16 hours. The plates were then chilled for 2 hours at +4°C, after which the membranes were punch-orientated using a sterile needle and removed from the plates. The plates were stored, inverted, at +4°C until required. The membranes were washed for 3 x 10 minutes in TBST before blocking non-specific binding sites by incubation in blocking buffer (section 2.2.7) overnight with constant gentle agitation. The plaque proteins were probed and any positives visualised as stated in section 2.2.10, using the sheep anti-*P. ovis* S1/S2 antisera at a

working dilution of 1:1000. Positive plaques were “cored” from plates and subjected to rounds of re-screening to allow the establishment of plaque-pure immunopositive stocks.

2.5.10 Differential Immunoscreen

Forty-eight clones were recognised by anti-*P. ovis* S1/S2 sera, therefore in order to determine which of these might be involved in the host protective immune response, a differential immunoscreen was carried out. Plaque-pure phage stocks of each of the 48 clones were screened using sera obtained from two animals that were strongly protected from mite challenge by vaccination with the S1 or S2 mite antigen with sera from two animals that were weakly protected. The antisera were found to cross-react with *E. coli* proteins and so were pre-absorbed prior to use (section 2.2.9). Those clones that were recognised by the sera from the strongly protected individuals were selected for further study as they may be involved in the host protective immune response.

2.5.11 *In vivo* Excision of Differentially Recognised Positives

Prior to obtaining DNA sequence data for each of the differentially recognised positive plaques, the plasmid pTriplEx2 containing the cDNA insert was excised from the phagemid using *in vivo* excision as described in the manufacturer’s instructions (Clontech, UK).

LB broth media (10 ml) was supplemented with 10 mM MgSO₄ and inoculated with a single colony of BM25.8 *E. coli* cells, which had been grown on LB/kan/cam plates (section 2.4.8). The culture was incubated at 31°C for 6 – 7 hours until the OD₆₀₀ was between 1.1 and 1.4. One hundred µl 1M MgCl₂ was added, and 200 µl of the culture fluid was combined with 150 µl of the plaque-purified differentially recognised positive phage stock. The samples were incubated at 31°C for 30 minutes without shaking, after which 400 µl LB broth was added. The samples were incubated for a further hour at 31°C with shaking. The infected cell suspension (1–10 µl) was then spread onto LB/amp plates and incubated overnight at 31°C.

Two transformants were selected for each of the differentially selected positive plaques and a liquid culture prepared as described in section 2.4.13, using LB broth supplemented with ampicillin. The DNA was purified from the liquid cultures using the Wizard *Plus* SV Minipreps System as described in section 2.4.14.

2.5.12 Sequencing of Differentially Screened Positives

The sequence of the insert DNA in the differentially selected positives was determined by the Functional Genomics Unit at Moredun Research Institute using Big-dye terminator chemistry (Applied Bio systems, UK) and a 377 XL DNA Sequencer (Applied Bio systems, UK).

The sequencing reaction uses a combination of PCR and Sanger chain termination techniques. A single primer is used in the presence of chain-terminating nucleotides to amplify the sequence of interest. The chain-terminators (dideoxynucleotides (ddNTPs)) have no free -OH group at the 3' end so that the chain cannot extend any further after their incorporation. Each of the nucleotides, A, T, G, and C, are represented by ddNTPs and each has a different fluorescent dye. The PCR reaction consisted of 1 µl of 3.2 pmol/µl sequencing primer, 2-5 µl target DNA (depending on concentration), 4 µl of Terminator Ready Reaction™ mix and dH₂O to a final volume of 10 µl. The Terminator Ready Reaction™ contained ddNTPs, dNTP's, *Taq* DNA Polymerase, MgCl₂, and Tris buffer, pH 9.0. The PCR conditions were 96°C for 10 seconds followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and finally 60°C for 4 seconds. Each PCR reaction produced a range of different fragments. The reaction was fractionated on a 5% polyacrylamide gel, and the sequence read from the order in which the reaction products were located on the gel. The sequencing primers used for these reactions were either:

5' sequencing primer: 5'-TCC GAG ATC TGG ACG AGC-3'

3' sequencing primer: 5'- TAA TAC GAC TCA CTA TAG GG-3'

5'screening primer: 5'-CTC GGG AAG CGC GCC ATT GTG TTG G-3'

all of which were provided with the Clontech Kit or the standard primers T7 and M13 forward and reverse.

2.5.13 Analysis of Sequence Data

The DNA sequences were initially analysed using the EditSeq programme of the DNASTar software package (Lasergene, DNASTAR Inc, <http://www.dnastar.com>). Any ambiguous base calling was edited using the SeqMan programme, where all sequence data available is aligned and ambiguous bases corrected. Database searches for homologues were carried out using the WU-Blast database on the EBI website (<http://www.ebi.ac.uk>). Alignments were obtained using the Clustal W algorithm from the EBI website or the Clustal X windows programme (Thompson, Gibson, Plewniak et al., 1997) and viewed using the GeneDoc programme (Nicholas & Nicholas, 1997).

2.6 EXPRESSED SEQUENCE TAG (EST) ANALYSIS

2.6.1 Plaque Selection and Sequencing

This work was carried out by staff at Dr. M. Blaxter's lab at the University of Edinburgh. Aliquots of the cDNA library were plated out as in section 2.5.3 using the blue/white colour selection to differentiate between recombinant and non-recombinant plaques. Five hundred recombinant plaques were randomly selected and phage stocks prepared from each, as described in section 2.5.4. Insert DNA was amplified using the following primers:

M13R forward primer 5'-AGCGGATAACAATTTACACAGGA-3',

T7PL reverse primer 5' – CTCACTATAGGGCGAATTGG – 3' and the PCR products sequenced using the primer TEXTF1 5'-CTCGGGAAGCGCGCCATTGT-3' which is designed from the λ TriplEx2 vector.

Insert cDNAs >500 bp were selected for sequencing. The PCR products were cleaned and sequence analyses conducted using ABI rhodamine dye terminators and an ABI 377 automated sequencer (Daub, Loukas, Pritchard et al., 2000). Sequences were checked and all vector and poor 3' sequences were removed. The edited sequences were compared to the public databases (GenBank non-redundant nucleotide and protein databases and dbEST) using the BLAST algorithms (Altschul, Gish, Miller et al., 1990), clustered on the basis of homology using CLOBB v1.0 (Parkinson, Guiliano, & Blaxter, 2002) and putative function assigned where

possible. All procedures were conducted within the quality control parameters outlined by Daub et al. (2000).

2.6.2 Recovery of Clones from Glycerol Stocks

After preliminary analysis of the EST sequence data, approximately 25 clones were selected for further analysis. These clones were selected on the basis of their homology to known allergens, proteases or free-radical scavenging enzymes. Dilutions (neat, 1:10, 1:100) of each phage stock were mixed with 200 µl of XL1 blue cells and the mixture plated out as described in section 2.5.3. Three plaques were selected for each clone of interest and a phage stock made as described in section 2.5.4.

2.6.3 PCR and Sequence Analysis of EST Clones

EST clones were analysed by PCR (2.5.5) to establish the presence and size of inserts. To remove excess primers, dNTPs or enzyme prior to DNA sequencing, DNA from each of the PCR reactions was purified from agarose gels, as described in section 2.4.16. The sequences of the purified PCR products were determined using the 5' and 3' sequencing primers supplied with the cDNA library construction kit (Clontech). The sequences for these primers are shown in section 2.5.12.

2.7 SEQUENCE ANALYSIS OF CYSTEINE PROTEASE ESTs

2.7.1 Generation of Full-length Sequence

ESTs encoding *Der f* 1 (accession number BQ834853), cathepsin L (BQ834906) and cathepsin B (BQ834852) homologues, respectively, were selected for further analysis. Full-length cDNA sequences were derived using PCR and gene-specific sense or antisense primers (Table 2.7) in combination with the 5' and 3' sequencing primers (section 2.5.12) with aliquots of the amplified cDNA library as target. PCR products were purified as described previously (section 2.4.15) and subcloned into the pGEM-T Easy vector (Promega, UK) according to the manufacturer's instructions.

EST homologue in parenthesis	Primer Name	Primer Sequence (5' to 3')
BQ834852 (<i>Der f 1</i>)	P1 sense	GAA ACA ACT GGA AAA GGT CGC ATC AA
	P2 sense	GCT ACA GTC GAA TCA ACA TAC TTA G
	P3 sense	CGA GCT CGC GCC GGA AGA TGT C
BQ834906 (cathepsin L)	P4 antisense	GTT TAC GCC AGT CAA CTT CTT CAG
BQ834913 (cathepsin B)	P5 sense	GTG CAA ATT CAT GGA ACG AAG ATT GG
	P6 antisense	GCA ACA ACT GCT CTT TCC ATA CCG CC

Table 2.7. Gene-specific primer sequences for each of the ESTs of interest.

2.7.2 Sequence Analysis

Sequence analysis was performed using the Editseq and SeqMan programmes of the DNASTar software, which allowed all parts of the sequence to be aligned. Once full-length sequence had been obtained, the sequence was first analysed using the WU-Blast database search on the EBI website to find homologues. An alignment of homologous sequences was then carried out using the Clustal W programme on the EBI website. The protein sequences were also analysed to determine any conserved motifs or domains of interest, using the software on the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) and Protein Information Resource (PIR) Pattern Search (<http://pir.georgetown.edu/pirwww/search/patmatch.html>) websites, which recognise protease active sites, glycosylation sites and signal peptides.

For the *Der f 1* homologue a limited phylogenetic analysis was carried out to determine which class of cysteine proteases this allergen belongs to. Previous studies have suggested that it is a cysteine protease (Chua et al., 1988; Stewart, 1995), but the type of cysteine protease has not been determined. An extensive phylogenetic tree, using sequences from all types of cysteine proteases, was recreated as described in Tort et al., (1999) with the house dust mite allergens, *Der p 1*, *Der f 1* and *Eur m 1* and the *P. ovis* homologue included. Initially, a sequence alignment of all of the sequences was prepared using the Clustal X programme (Thompson et al.,

1997). The alignment was then analysed using the Treeview programme (Page, 1996) which performs phylogenetic analysis using the Phylip algorithm.

CHAPTER 3: PROTEASE CHARACTERISATION

3.1 INTRODUCTION

Due to the development of acaricide-resistant mites and consumer concern regarding drug residues in meat products novel mechanisms to control sheep scab are currently the focus of research. Sheep develop a strong protective immunity to re-infection (Bates, 2000; van den Broek et al., 2000) and this response can be experimentally induced by vaccination with parasite extract in cattle (Losson, Detry-Pouplard, & Pouplard, 1988), and sheep (Smith et al., 2002); suggesting that vaccination may be a suitable control strategy against *P. ovis* infestation. *P. ovis* mites can ingest and cleave host IgG, an activity that may be due to protease activity (Pettit, Smith, Richardson et al., 2000). Proteases are considered essential for parasite survival and have also been described as potential immunomodulators and important allergens. The presumed functions of proteases in parasite feeding, tissue penetration and evasion of host immune responses have led to their purification and evaluation in protection trials. To date, there have been several experiments conducted in nematodes and fluke that have shown that proteases are effective as vaccine candidates. The immunisation of cattle with cathepsin L1 from the trematode, *Fasciola hepatica*, (Dalton et al., 1996a) resulted in significant levels of protection. Aminopeptidases have also been shown to give significant levels of protection, for example, immunisation with the aminopeptidase, H11, from *H. contortus* resulted in significant levels of protection in sheep (Smith, Munn, Graham et al., 1993). Moreover, vaccination of sheep with leucine aminopeptidase elicited 89% protection against fasciolosis (Piacenza, Acosta, Basmadjian et al., 1999). In addition, proteases partially purified from water-soluble and membrane extracts of adult *H. contortus* are potentially useful protective immunogens (Boisvenue et al., 1992; Knox, Smith, Smith et al., 1995; Knox, Smith, & Smith, 1999). The latter substantially protected lambs against a single challenge infection with *H. contortus* with daily faecal egg outputs reduced on average by 77% and final worm burdens by 47% in three trials (Knox, Smith, & Smith, 1999).

Many of the above mentioned proteases have been localised to the parasite gut. Targeting gut membrane proteins as vaccine components was pioneered in ticks and this work led to the launch of a commercial vaccine against *Boophilus microplus*, the Australian cattle tick (Willadsen et al., 1995).

Previous studies on *P. ovis* mites suggest that they contain a variety of protease activities. To date, the major endoprotease of *P. ovis* has been identified as an aspartic protease (Nisbet & Billingsley, 1999b). The authors suggest that this protease may be an important digestive enzyme and may also cause the hypersensitivity response observed on infected animals. Also identified was leucine and valine aminopeptidases but throughout these studies no serine proteases were identified. Further analyses identified a 28 kDa cysteine protease using a biotinylated probe (Nisbet & Billingsley, 2000). The proteases present in *P. ovis* may be valid vaccine candidates because, as described earlier, they may be essential for the survival of the parasite. However, before vaccine candidates can be identified there is a need to determine the nature of the proteases present. The aim of this study was to define the range of proteases present in whole extracts of *P. ovis* and to examine their ability to degrade a range of protein substrates that may be encountered in the mites' natural habitat.

3.2 RESULTS

3.2.1 Substrate gels

Protease activity was initially sought using protein substrate gels, which are polyacrylamide gels co-polymerised with the protein of choice.

Substantial protease activity was visible in the pH range 3-11 with gelatin as substrate (Figure 3.1). At acidic and neutral pH the activity of some of the proteases was enhanced by the addition of DTT (Figure 3.1, see pH 3-6). Proteolysis was visible over a wide size range, from 35 kDa to > 205 kDa, and appeared to consist of several poorly resolved zones (Figure 3.1, see pH 4). Three distinct bands of proteolysis were however evident at 65, 55 and 50 kDa over a broad pH range (pH 4-11) with optimal activity at pH 6. The enzyme activity was almost completely inhibited by the cysteine protease inhibitor, E64 (Figure 3.2A, lane 3). However, the three distinct bands were fairly insensitive to the inhibitors tested but activity was reduced slightly by the metallo-protease inhibitor, 1, 10 phenanthroline, at pH 9 (Figure 3.2B).

When azocasein was used as substrate in the gels, activity was very much reduced compared to gelatin gels. A faint smear of activity was present when DTT was included, and only faint bands at 45 and 66 kDa were evident without DTT. These bands were sensitive to E64, in both the S1 and S2 extracts at pH 6 and 7 (not shown). No activity was observed with haemoglobin as substrate. A triplet of bands similar to those in gelatin gels was evident, although not as strongly, with fibrinogen as substrate, at pH 5 and 6. An additional doublet was observed at 44 and 38 kDa with optimal activity at pH 6 (Figure 3.3). The triplet, but not the doublet, was sensitive to inhibition by 1, 10 phenanthroline.

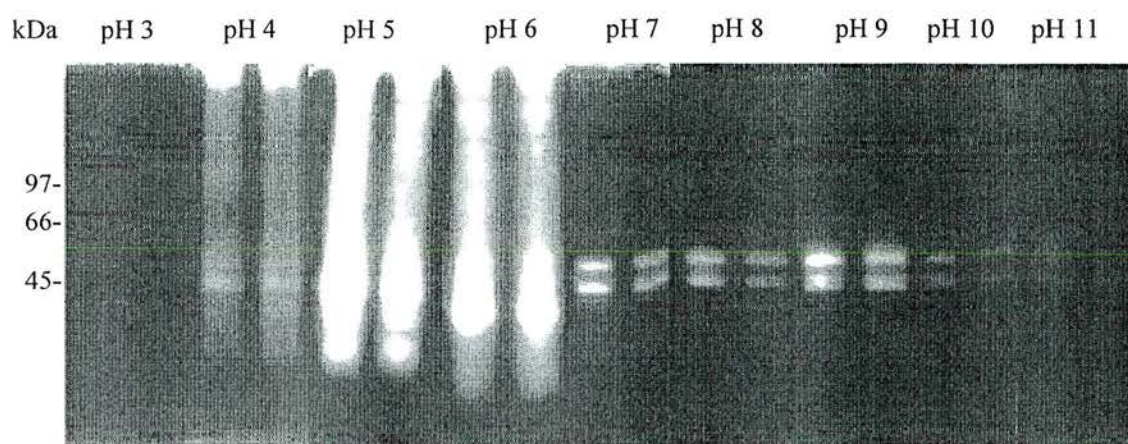


Figure 3.1 Gelatin substrate gel showing the enzymatic activity of the S1 and S2 extract of *P. ovis* over a range of pH values. The S1 extract is shown on the left lane of each pair and the S2 extract is shown on the right. DTT (2 mM) was included in pH 3 to pH 6 buffer.

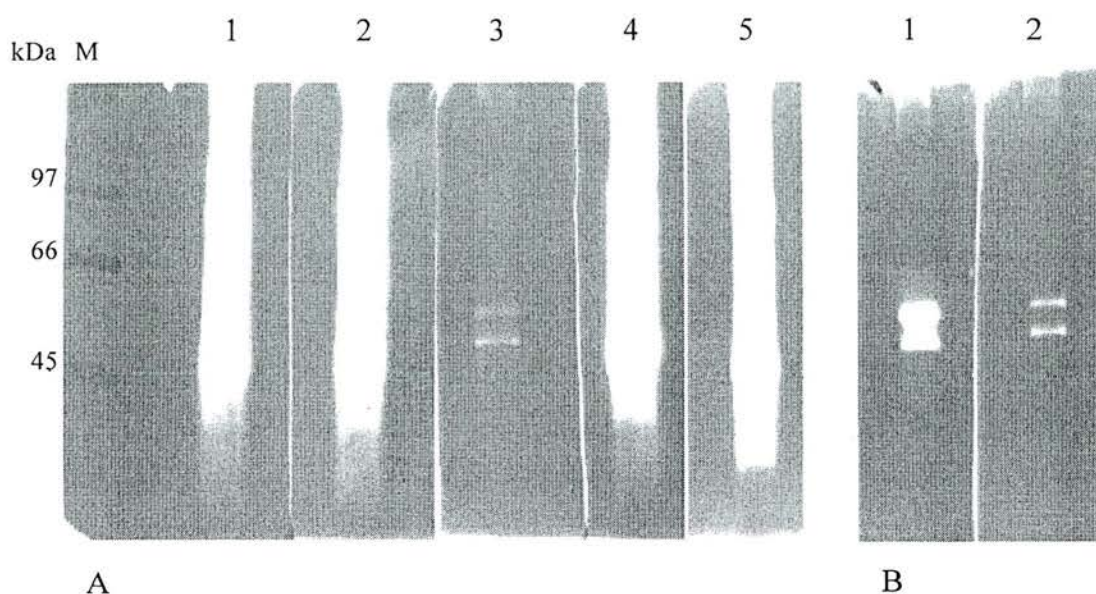


Figure 3.2 Gelatin substrate gel. **Panel A:** at pH 5 with 2 mM DTT. M; molecular weight markers, Lane 1; control, mite extract alone, lane 2; mite extract with 2 mM AEBSF, lane 3; mite extract with 10 μ M E64, lane 4; mite extract with 10 μ M pepstatin A, lane 5; mite extract with 1mM 1, 10 phenanthroline. **Panel B:** pH 9, lane 1; control, mite extract alone, lane 2; mite extract with 1 mM 1, 10 phenanthroline.

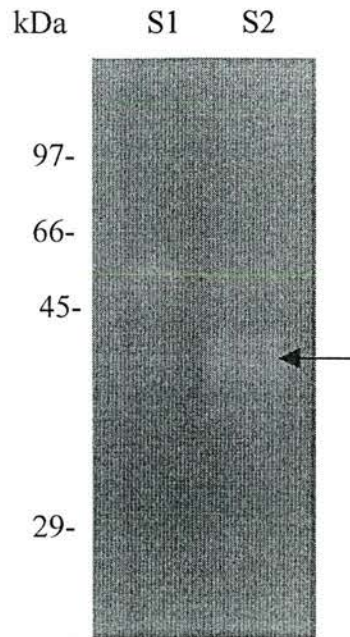


Figure 3.3. Fibrinogen substrate gel at pH 6 with S1 and S2 extracts. An additional doublet was clearly evident in S2 extracts (arrow) at approx. 38 and 40 kDa, which was not present in gelatin substrate gels.

3.2.2 Degradation Assays

To complement the substrate gel analyses, protein degradation by mite extracts was sought by incubating selected protein substrates directly with mite extracts and visualising the outcome of the reactions using SDS-PAGE and Coomassie Blue staining. While this assay does not provide information on the molecular size of the proteases involved in digestion, it is more sensitive and allows cleavage products to be visualised. Throughout these degradation assays similar profiles were obtained by the S1 and S2 extract, therefore the figures show an example of the degradation by one or the other and are labelled S1/S2.

Haemoglobin

Optimal degradation of haemoglobin occurred at pH 3, and was sensitive to inhibition by the aspartyl protease inhibitor, pepstatin A (Figure 3.4). Degradation was unaffected by other inhibitor classes.

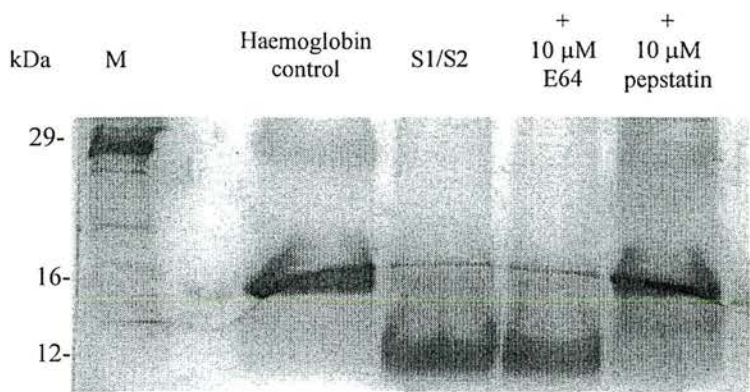


Figure 3.4 Haemoglobin degradation assay at pH 3 with 2 mM DTT. M, molecular weight markers

Fibrinogen

All three fibrinogen peptide chains were completely degraded (Figure 3.5A) at pH 4 and 5 with no degradation evident above pH 5. Degradation was abolished by pepstatin A and partially reduced by 1,10 phenanthroline.

IgG

IgG degradation occurred only at pH 4 and 5. The degradation was restricted to the heavy chain (~ 55 kDa) and resulted in a degradation product at 30 kDa (Figure 3.5B). Degradation was completely inhibited by 1,10 phenanthroline and E64 and faintly inhibited by pepstatin A and AEBSF.

Albumin

Degradation of albumin occurred only at pH 3 and was slightly inhibited by E64 and to a lesser extent by pepstatin A (Figure 3.5C).

Fibronectin

Fibronectin was degraded across a wide pH range (pH 3-9) by both the S1 and S2 extracts. Degradation was inhibited by pepstatin at pH 3, E64 and 1,10 phenanthroline at pH 5, and slightly inhibited by E64 at pH 9 (Figure 3.6).

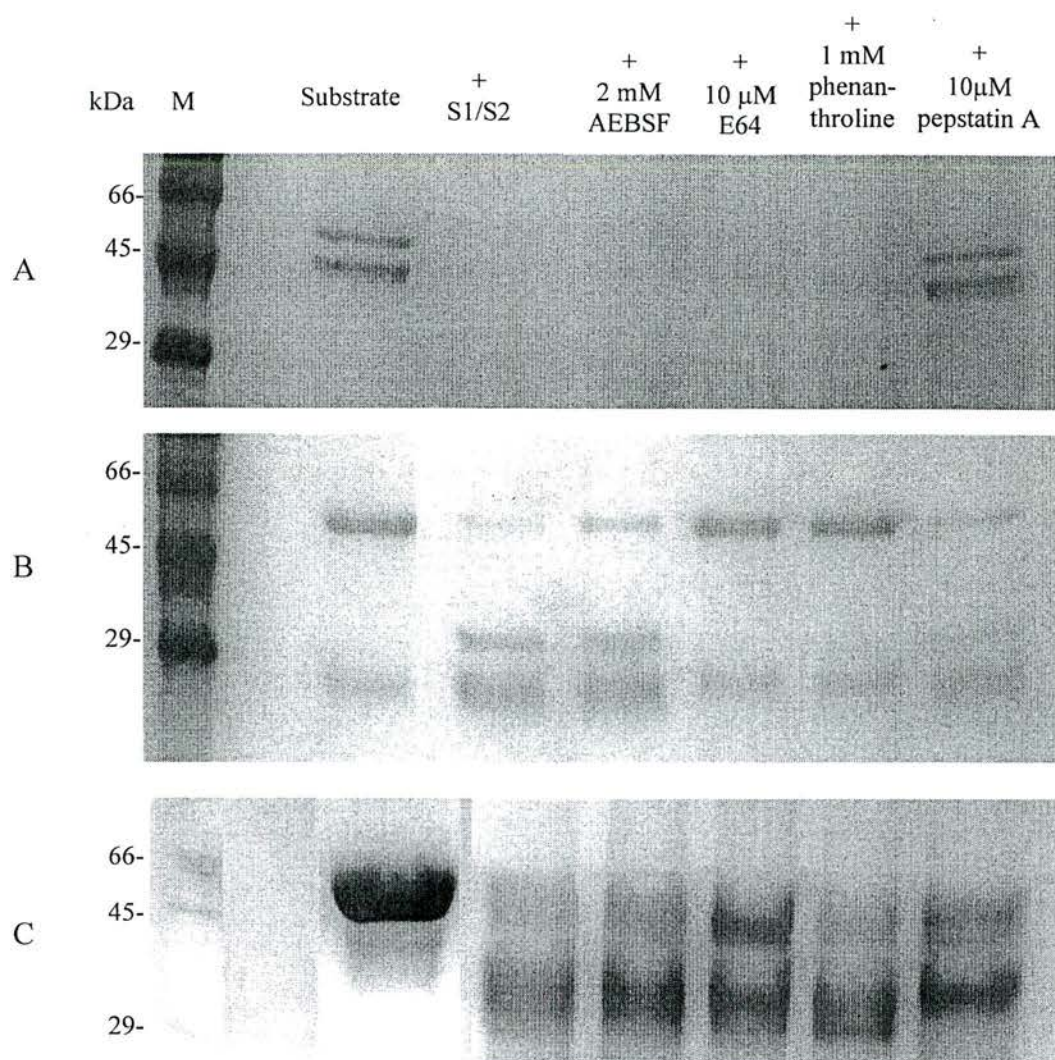


Figure 3.5 Degradation Assays – all samples included 2 mM DTT, M, molecular weight markers. **Panel A:** Fibrinogen degradation assay at pH 4, **Panel B:** IgG degradation at pH 4, **Panel C:** Albumin degradation at pH 3.

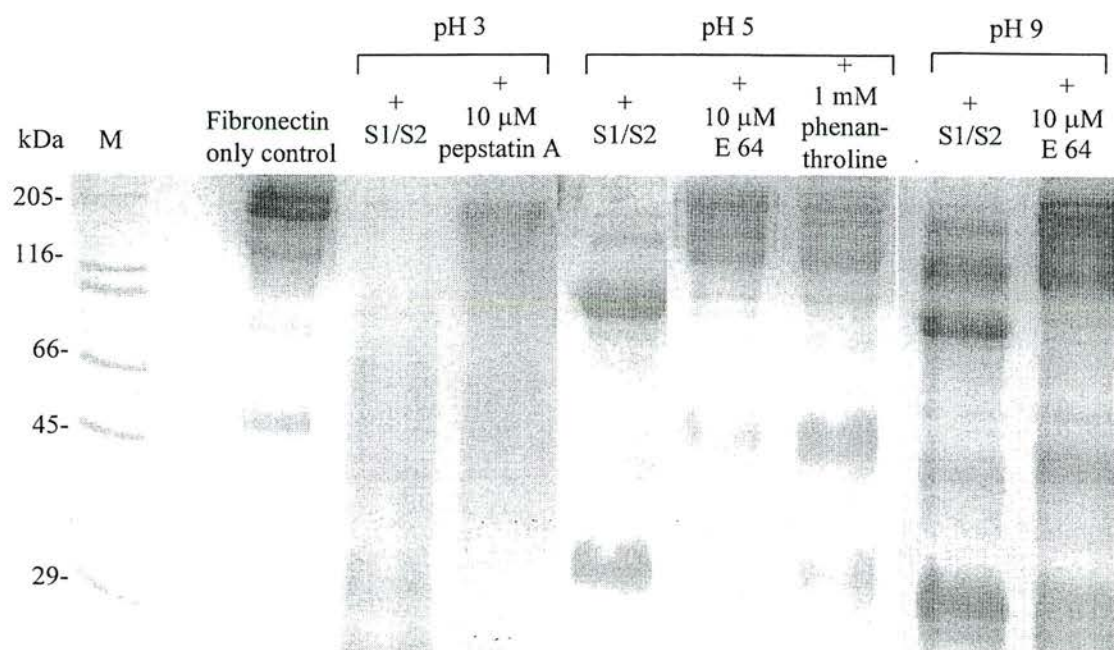


Figure 3.6 Fibronectin degradation assay over a range of pH values, M, molecular weight marker.

Azocoll, Elastin-orcein and Keratin-azure

Azocoll was degraded by both the S1 and S2 extracts, the optimum degradation was found to occur at pH 7 (Figure 3.7) and was inhibited by 1,10 phenanthroline. Keratin and elastin-orcein were not degraded by either the S1 or S2 extract.

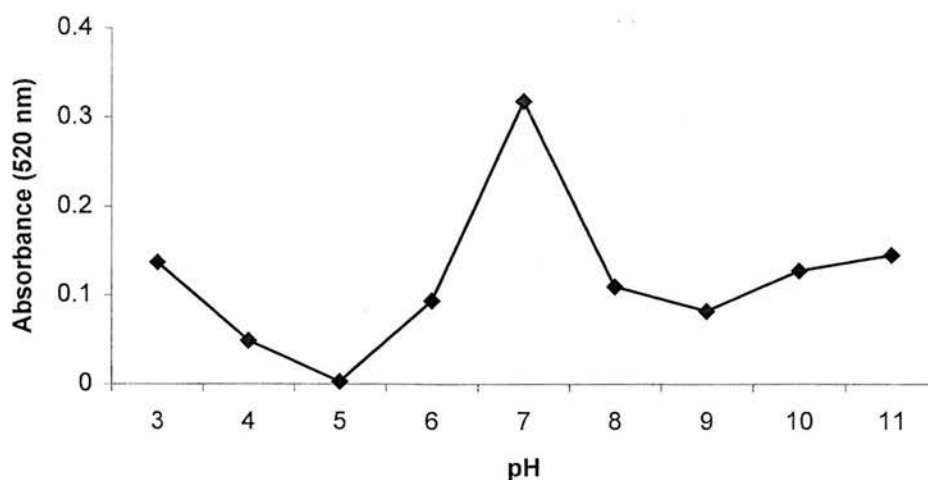


Figure 3.7 Azocoll degradation assay showing the pH optima. Each point on the graph represents the absorbance value at each pH level, minus the absorbance of a PBS control at an equivalent pH value.

3.2.3 Antibody Inhibition Assays

There were no differences observed between the control S1 and S2 extracts and those that had been pre-incubated with antibody purified from either 1) sera from immunised and protected animals, 2) naïve control sera or 3) infestation sera (S2 shown, see Figure 3.8).

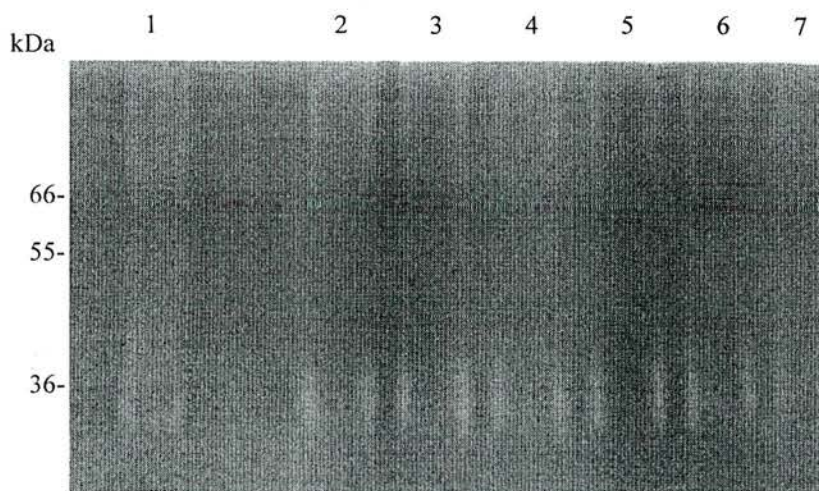


Figure 3.8 Antibody inhibition assay. Lane 1; S2 extract, Lanes 2-4; S2 extract and 5 μ g of the purified IgG from: lane 2; animals immunised with S1 or S2, lane 3; naïve control animals, lane 4; infestation sera. Lanes 5-7; S2 extract and 10 μ g purified IgG from: lane 5; animals immunised with S1 or S2, lane 6; naïve control animals, lane 7; infestation sera.

3.2.4 Acetylcholinesterase Assays

No esterase or acetylcholinesterase activity was identified in either the S1 or S2 extracts by either the gel based or spectrophotometric assays (results not shown).

3.3 DISCUSSION

The aim of this study was to determine the range of proteases active in *P. ovis* water-soluble (S1) and membrane associated (S2) mite extracts and to determine the nature of the substrates degraded. The substrates were chosen on the basis of their possible occurrence in the natural mite habitat on the host. This study showed that the S1 and S2 fractions have very similar protease profiles (Figure 3.1) with a range of proteases present that are capable of degrading a wide variety of substrates.

Cysteine protease activity was found to dominate at acidic pH (Figure 3.1), as indicated by inhibition with E64, a cysteine protease specific inhibitor (Barrett, Khembhavi, Brown et al., 1982; Figure 3.2). The cysteine proteases were capable of degrading several substrates that are commonly found in the skin, for example, gelatin, azocoll and fibronectin. Gelatin and azocoll are soluble components of collagen whilst fibronectin is an extracellular adhesion glycoprotein found in connective tissue and on cell surfaces. Metallo-proteases capable of degrading gelatin were also present, as shown by 1, 10 phenanthroline inhibition (Figure 3.2B). The proteolysis of these substrates may aid in the penetration of the skin, or in the initiation of the lesion. This function has been ascribed to proteases of other parasitic organisms. For example, metallo-proteases of the hookworm, *Ancylostoma* spp. have been implicated in tissue migration (Hotez, Haggarty, Hawdon et al., 1990). In addition, the cysteine proteases of *Fasciola hepatica*, cathepsin L1 (Smith, Dowd, McGonigle et al., 1993b) and cathepsin L2 (Dowd, Smith, McGonigle et al., 1994), degrade a variety of extracellular matrix and basement proteins and are thought to be important in tissue penetration (Berasain, Goni, McGonigle et al., 1997).

It is possible that the proteases present in *P. ovis* may have an anti-coagulant function as determined by the degradation of fibrinogen and albumin. Fibrinogen degradation was attributable to aspartyl and metallo-proteases (Figure 3.3 and 3.5A). Albumin degradation occurred at an acidic pH, and was due to cysteine and aspartyl proteases (Figure 3.5C). It is feasible that these substrates would be present in the mite habitat, as the hypersensitivity response results in the leakage of serum proteins onto the skin surface of infested animals (Stromberg & Fisher, 1986). Degradation of these substrates may prolong the release of serous exudate from the lesion thus

increasing feeding opportunities for the mite, especially if the proteases responsible are secreted. The location of these proteases *in vivo* is, as yet, undefined but this information, when available, will help to confirm an extracellular function. It is likely that some or all of these proteases are located in the salivary glands, or the digestive tract of the mites. All were found in the water-soluble fractions suggesting that they are not bound to integral membranes within the mites.

In agreement with the observations of Nisbet & Billingsley (1999b), haemoglobin degradation was found to occur at acidic pH and was due to aspartyl protease activity (Figure 3.4). Although haemoglobin is not thought to be a major part of the mite diet (DeLoach, 1984), it may provide a source of useful nutrients when available. Nisbet and Billingsley (1999b) suggested that the aspartyl protease might be the major digestive endoprotease of the *P. ovis* mites. The present study expands these observations by providing evidence that cysteine and metallo-proteases may also be important for digestion.

Keratin and elastin were not degraded by the *P. ovis* proteases. This may reflect the fact that mites browse on the skin surface and do not require penetrating mechanisms. Mite protease may disrupt skin integrity by hydrolysing fibronectin thus loosening intercellular junctions. Other skin penetrating parasites have been found to contain proteases that degrade keratin and elastin e.g. chymotrypsin-like protease from *Schistosoma mansoni* (Salter, Lim, Hansell et al., 2000). However, keratin-containing skin squames are thought to be a major part of the house dust mite diet (Stewart, Lake, & Thompson, 1991) but whether these species can actually degrade keratin remains to be determined. Comparative analyses of the proteases found in different mite species is likely to provide useful functional information that can be used to elucidate their *in vivo* function.

The potential importance of cysteine and metallo-proteases in the physiology of the mite is underlined by their involvement in IgG degradation (Figure 3.5B). This supports and considerably expands on earlier observations that *P. ovis* ingests and degrades IgG (Pettit et al., 2000). The digestion of IgG was restricted to the heavy IgG chain, which may indicate a specific attempt to inhibit function. For simple nutrient acquisition, it might be expected that both chains would be equally degraded. Previous work has shown that other parasites also degrade IgG, e.g.

excretion/secretion products of *Fasciola hepatica* were found to degrade IgG (Berasain, Carmona, Frangione et al., 2000). The authors suggested that immunoglobulin degradation might aid in the evasion of the host immune response. Immune evasion through degradation of host IgG could occur in helminth parasites, which are usually surrounded by host tissue. Whether similar reactions occur in ectoparasites, which may not be enveloped by host tissue, still remains to be determined. However, the ability to degrade immunoglobulin could enhance mite survival *in vivo*. For example, the serous exudate, which is released from the skin of infested animals, may contain host antibodies. Previous studies have shown that animals produce *Psoroptes* specific antibody in response to infestation (Fisher, 1972; Wassall et al., 1987), and it has been suggested that antibody can damage parasites (Sinclair & Filan, 1991), therefore if the mites were able to degrade antibody, then they might be able to protect themselves from the host immune response.

Proteases identified from the house dust mite, *Dermatophagoides* spp. are known to be highly allergenic in the host and it is thought that their protease function may lead to an increase in allergenicity (Hewitt, Foster, Phillips et al., 1998). The group 1 allergens of the house dust mites are known to be cysteine proteases and are found in whole body and faecal extracts of mites (Thomas, Heap, & Carswell, 1991). They are thought to degrade skin collagens and keratin and may be the major digestive enzyme of this species (Colloff, 1993). Nisbet & Billingsley (2000) identified a 28 kDa cysteine protease from *P. ovis* using a biotinylated probe. The present study provides evidence that mite extracts contain more than one cysteine protease and it is possible that one or all of these are homologous to the group 1 allergens and hence may contribute to the pathogenesis of sheep scab.

Other major allergens of house dust mites are the serine proteases, group 3, 6 and 9 allergens (Stewart, Kollinger, King et al., 1994). However, work carried out by other investigators (Nisbet & Billingsley, 1999b; 2000), has not identified any serine protease activity in *P. ovis* extracts. This study cannot confirm these findings as the irreversible serine protease inhibitor, PMSF, was added to the mite extraction buffer. PMSF also inhibits AChE, therefore the lack of AChE activity in the present study may be misleading and may instead reflect the inhibition of any AChE activity. EDTA was also added to the initial extraction buffer. It is a chelator of metal ions

and a reversible metallo-protease inhibitor. In order to define the types of metallo-proteases present in *P. ovis*, the activity could be inhibited with EDTA, then the proteases activated by the addition of specific metal ions. If protease activity was detected this would suggest the type of metallo-protease present. The mite extract used here was originally prepared for use as antigen in vaccine trials, and due to the shortage of mites, fresh extracts could not be prepared. In the future it would be interesting to prepare extracts without inhibitors present and repeat the protease activity assays.

Aspartyl proteases have not been identified as allergens in house dust mites but the cockroach (*Blattella germanica*) allergen, *Bla g 2* shares homology with aspartic proteases (Arruda, Vailes, Mann et al., 1995). This allergen is thought to be a major digestive protease in the cockroach. Nisbet & Billingsley (1999b) suggested that the *P. ovis* aspartyl protease might be responsible for the hypersensitivity response observed in affected animals.

In order to determine if any of the proteases are implicated in the immune response to natural infestation as well as that induced by vaccination with mite extracts, antibody binding to mite proteases was evaluated using an electrophoretic inhibition/mobility assay (Todorova, Knox, & Kennedy, 1995). Sera from sheep with a current infestation of *P. ovis*, or from sheep immunised and protected against challenge with the S1 or S2 mite extracts, recognises many proteins in both the S1 and S2 extract (Smith et al., 2002). Therefore, antibody was harvested from these animals, then reactivity with mite extract proteases was compared with antibody from uninfected controls. No differences were observed in electrophoretic mobility or enzyme activity (Figure 3.8), suggesting that proteases were not inhibited by incubation with antibody. Antibody inhibition of protease activity has been described previously, for example, Knox & Kennedy (1988) showed that the *in vitro* released proteases of the nematode, *Ascaris suum*, were inhibited by IgG antibody purified from rabbits infected with *A. suum* eggs. Furthermore, the excretory/secretory (ES) products of the parasitic nematode, *Trichinella spiralis*, were found to contain many proteases that were inhibited by antibody purified from immune mice, but not from naïve control animals (Todorova, Knox, & Kennedy, 1995). In both of these studies, the authors suggested that the host antibody response to the proteases and its action

in inhibiting the proteases might be a protective host mechanism to limit the damage caused by these parasites. While no effects on enzyme activity or mobility were observed in this study, this does not rule out the possibility that these proteases are targets for the host immune response. More detailed analyses would be required using much more sensitive techniques such as ELISA with purified native proteases or recombinantly derived proteases.

Although in this study many possible functions for the proteases identified have been discussed, it should be noted that the protein degradation studies do not provide conclusive evidence for function *in vivo*. These studies can, however, provide information on the types of protease activities present, and their specificity for certain substrates, the degradation of which may be advantageous to the parasite. N-terminal amino acid sequence analysis and molecular weight determinations of the peptide fragments resulting from the action of a purified protease on a defined protein substrate would provide useful information on the specificity of the enzyme active site, which may have application for the development of novel and specific therapeutics.

This work has shown that there is substantial protease activity in *P. ovis* mites. These proteases are capable of degrading a wide variety of substrates, and they may have several functions within the mite. Future work is required to determine if these proteases provoke an immune response during the course of infection, particularly if they invoke an IgE response, which would implicate them in the immediate type 1 hypersensitivity reaction.

CHAPTER 4: COMPLEMENTARY DNA (cDNA) LIBRARY CONSTRUCTION AND IMMUNOSCREEN

4.1 INTRODUCTION

Studies have shown that the hypersensitivity reaction in response to *P. ovis* infestation can be produced artificially by injection with mite extract (Stella, Braun, & Nunez, 1997), which suggests that vaccination could be a feasible alternative control mechanism. Studies carried out at Moredun, have shown that fractionation of the mite extract, using a range of detergents (Smith et al., 2002) and lectin-binding affinity purification (W. D. Smith, Personal Communication), results in increased levels of protection against mite challenge. To date, the water-soluble (S1) and membrane associated (S2) mite extracts have proved to be the most promising vaccine candidates, resulting in a two-fold reduction in mean lesion areas, and a seven-fold reduction in mite numbers in treated compared to untreated control animals (Smith et al., 2002).

At present, a few limited studies have been carried out to identify the mite antigens that may induce the immune response observed in sheep. These studies have mainly focused on fractionating the mite extract by SDS-PAGE then carrying out Western blotting to identify the antigens present. Boyce & Brown (1991) identified at least 30 antigens present in *P. ovis* using sera from naturally infested bighorn sheep and Matthes et al. (1996) identified at least 24 antigens using sera from naturally infested sheep. Of these 24 antigens, 13 were recognised by a mouse anti-sheep IgE antibody, suggesting that these antigens could be associated with the observed hypersensitivity response to *P. ovis* mites. Jayawardena et al. (1998) using sheep antisera and crude extracts from mites raised on cattle (to reduce the chance of cross-reactivity) identified six antigenic bands, ranging from 12-183 kDa. Although these studies determined the number and size range of the antigens present, they do not allow any further characterisation of the nature of individual antigens.

There is a need to identify the antigens that evoke the hypersensitivity responses in sheep, to lead to a better understanding of the disease, and provide information that could be useful in the hunt for putative vaccine candidates. The aim

of this study was to generate a cDNA library from mixed stage *P. ovis* mites and screen it with sera from animals immunised with mite extract (Smith et al., 2002), to identify proteins that are associated with the protective immune response. As a second approach to identify mite proteins of interest, an expressed sequence tag (EST) analysis was also conducted, and the results are described in Chapter 5.

4.2 RESULTS

4.2.1 Messenger RNA (mRNA) Extraction

mRNA was extracted from approx. 500 mg mixed stage *P. ovis* mites using the Stratagene mRNA extraction kit, as described in section 2.5.1. The total yield of mRNA was 252 ng (6.3 ng/ μ l). The mRNA was fractionated on 0.8% agarose gel and viewed under UV. There was a faint smear of material present so, to increase sensitivity of the analysis, the mRNA was fractionated using 7.5% PAGE and visualised by silver staining. A smear of material ranging in size from over 2.0 kb to about 500 bp was present (Figure 4.1). A cDNA library was prepared using this mRNA and the SMART™ cDNA Library Construction Kit (Clontech Laboratories, UK) as described in Chapter 2 (section 2.5.2).

4.2.2 Titre of Primary and Amplified cDNA Libraries

The titre of the cDNA library was obtained by plating serial dilutions of the packaged cDNA library, as described in section 2.5.3. The titre of the primary library was 1.2×10^6 plaque forming units (pfu)/ml and the library contained 89% recombinant phage. The titre of the amplified library was 7.6×10^8 pfu/ml.

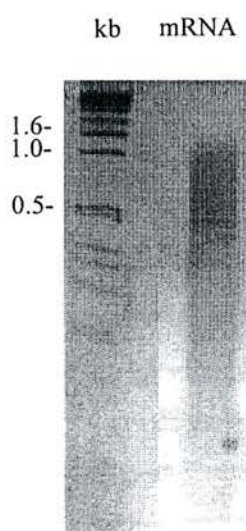


Figure 4.1. The *P. ovis* mRNA fractionated using 7.5% PAGE and visualised by silver staining.

4.2.3 Analysis of Insert Size

PCR analysis was carried out using vector primers on 20 randomly selected recombinant plaques and two non-recombinant plaques to determine the size of inserts present as described in section 2.4.5. A variety of insert sizes was observed with 15 of the 20 selected being above 500 bp in size (Figure 4.2). Of these inserts, eight were greater than or equal to 1 kb, and there were two plaques with insert sizes greater than 2.0 kb.

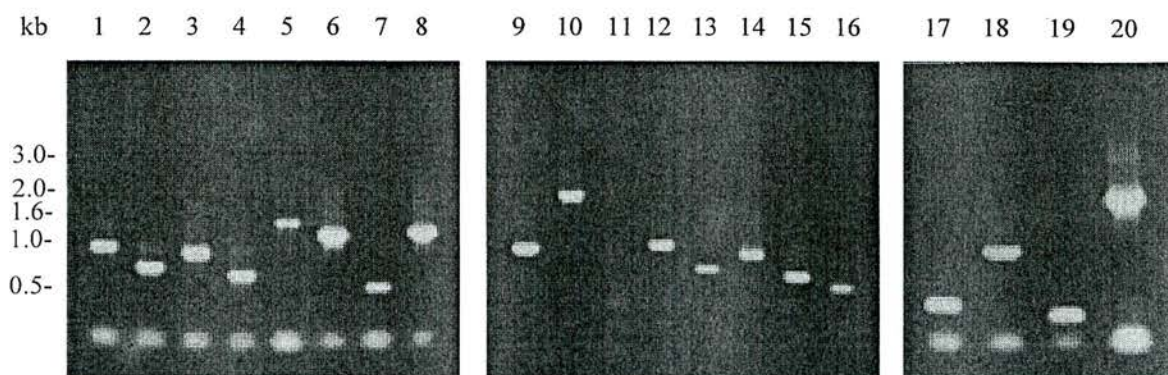


Figure 4.2. 0.8% agarose gels showing the cDNA inserts, amplified using PCR, from each of the 20 randomly selected recombinant phage (Lanes 1-20).

4.2.4 Western Blot of Mite Antigen Probed with Sheep Anti-*P. ovis* S1 and S2 Sera

A Western blot analysis was carried out to determine the molecular size range of the antigens recognised by the sheep anti-*P. ovis* S1 and S2 sera (S1 antigen shown, Figure 4.3). Many proteins were recognised by both the S1 and S2 sera and ranged in size from >205 kDa to < 21 kDa, with a limited number of prominent bands evident. Since the antigen recognition profiles of the two sera were essentially the same the two sera were pooled for future use. Mite antigen also reacted with the secondary antibody alone (lane 2, Figure 4.3) and the same profile was observed with the negative control serum (lane 3, Figure 4.3).

The optimum dilution of the primary antibody for library immunoscreening was determined to be 1:1,000 (results not shown), a dilution at which the positive plaques would be clearly distinguishable from non-recombinant plaques. The sera did not cross-react with *E. coli* or phage proteins.

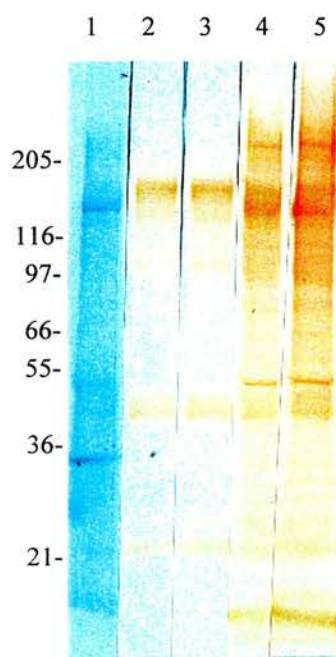


Figure 4.3. The Western blot of mite antigen (S1) probed with sheep anti-*P. ovis* S1 or S2 sera at 1: 500 dilution. Lane 1: Coomassie stained S1 antigen; lane 2: antibody blank - no primary sera used; lane 3, negative control sera; lane 4, sheep anti-*P. ovis* S1 sera; lane 5 sheep anti-*P. ovis* S2 sera.

4.2.5 cDNA Library Immunoscreen

The cDNA library was screened with sheep anti-*P. ovis* S1/S2 antisera as described in section 2.5.9. Immunoscreening of approx. 140,000 plaques resulted in the identification of 48 positive plaques from the cDNA library. An example of the intensity of the positive plaques is shown in Figure 4.5.

4.2.6 Western Blot of Mite Antigen Probed with Antisera from Strongly or Weakly Protected Animals

In the vaccination trials described by Smith et al. (2002), there was considerable variation between individuals observed in the degree of protection conferred by vaccination. If some antigens were only recognised by protected individuals, these antigens would be worthy of further attention. However, the Western blot analysis (Figure 4.4) showed that, overall, there were no obvious

differences in the proteins recognised between the strongly and weakly protected individuals although the strength of the response to individual antigens varied.

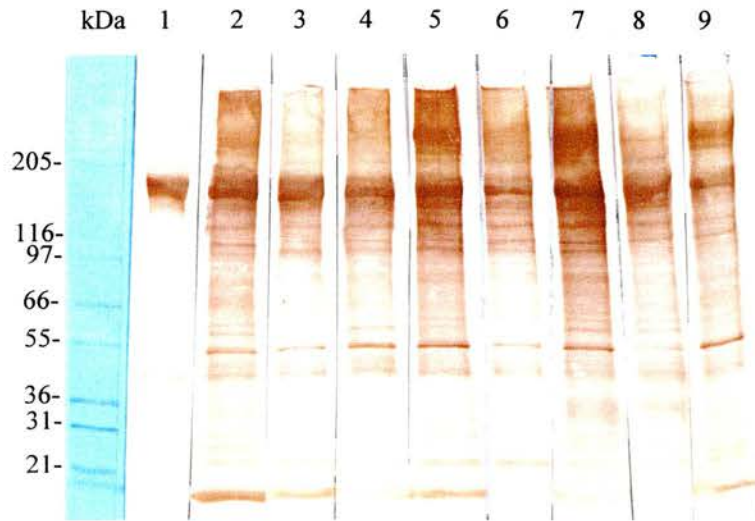


Figure 4.4 Western blot of the S2 mite extract probed with sera from either strongly or weakly protected sheep anti-*P. ovis* S1/S2 sera. Lanes 1: negative control sera, lanes 2-3: sera from highly protected sheep from S1 vaccination trial, lanes 4-5: sera from weakly protected sheep from S1 vaccination trial, lanes 6-7 highly protected sheep from S2 vaccination trial and lanes 8-9: weakly protected sheep from S2 vaccination trial.

4.2.7 Differential Immunoscreen

Forty-eight clones were recognised by the sheep anti-*P. ovis* S1/S2 sera, therefore in order to determine which ones may be associated with the host immune response, a differential immunoscreen was carried out (section 2.5.10). Those clones recognised by only highly protected individuals may be important in the protective immune response. The differential immunoscreen showed that six of the 48 positives were recognised by the strongly protected sera, but not recognised by the weakly protected sera, an example of the results obtained is shown in Figure 4.5. These positives were designated Po9, Po11, Po14, Po33, Po38 and Po41 with insert sizes ranging from 1 kb to 2.1 kb (Figure 4.6).



Figure 4.5 An example of the result of the differential immunoscreen on clone number Po 41. The left side of the picture shows the clone probed with the weakly protected sera, and the right side shows the clone probed with the strongly protected sera. Dark positive plaques are recognised by the strongly protected sera.

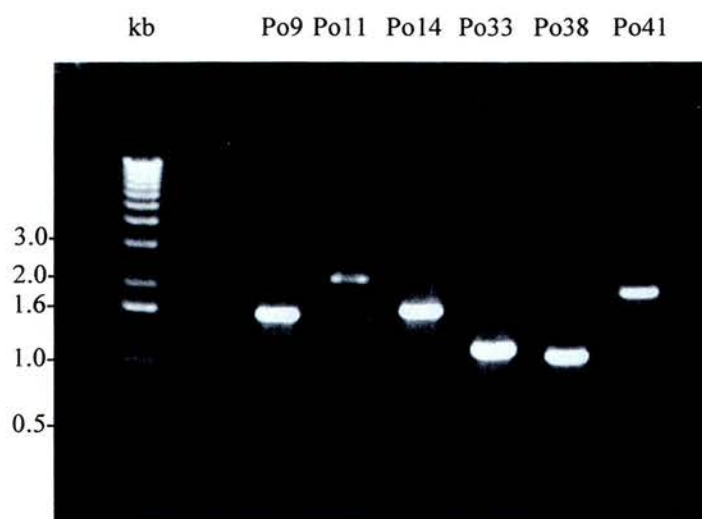


Figure 4.6 Insert DNA, amplified for each of the differentially selected positive plaques by PCR, fractionated in a 0.8% agarose gel with ethidium bromide and visualised using UV illumination.

4.2.8 Sequencing of Clones Recognised by the Differential Immunoscreen

DNA sequence analysis showed that five of the clones, Po9, Po11, Po14, Po33 and Po38, shared similar degrees of homology at the amino acid level (35% identity $e = 1.4^{-25}$ to 1.8^{-31}) with catchin, a myosin-like protein from the Mediterranean mussel, *Mytilus galloprovincialis* (GenBank accession number Q9U0S5). The alignment of the deduced protein sequence of the *P. ovis* clones to the 5' end of the catchin protein is shown in Figure 4.7.

The deduced protein sequence for the remaining positive, Po41, encoded an open-reading frame of 152 amino acids but showed no significant homology to any sequences in the databases. The nucleotide and amino acid sequence for Po41 is shown in appendix 1.

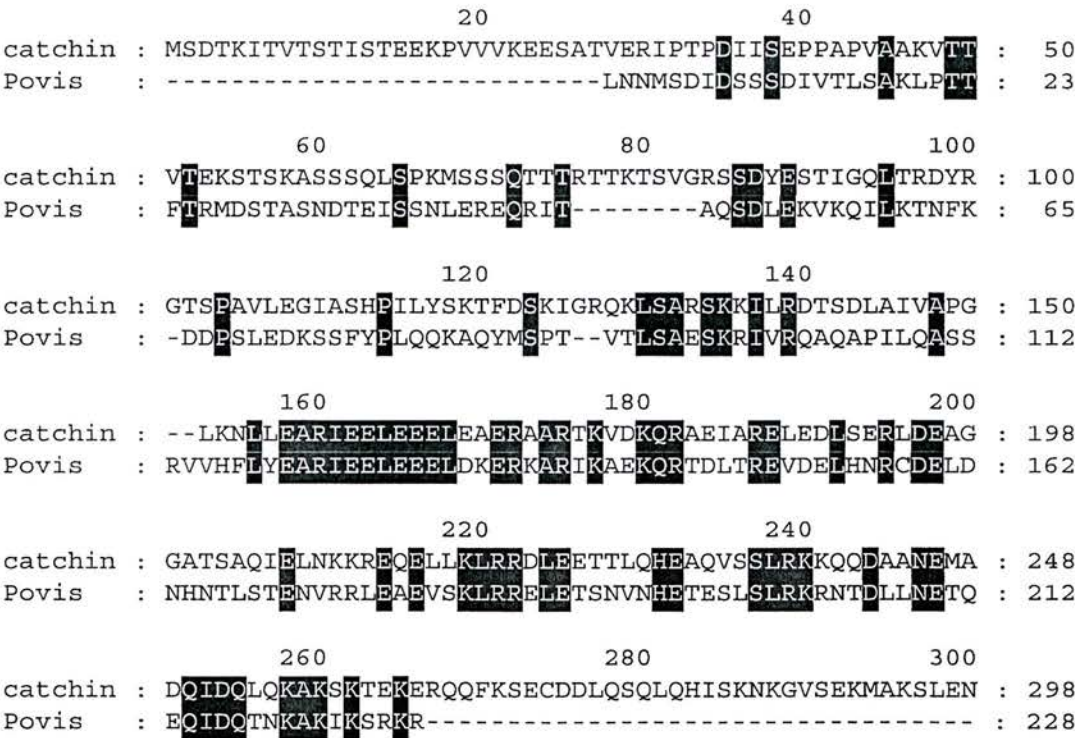


Figure 4.7 The alignment of the 5' end of the catchin sequence with the sequence of the clones selected by the differential immunoscreening. Residues in black are 100% identical. The catchin sequence has been curtailed at residue 298 for the purposes of this diagram.

4.3 DISCUSSION

The cDNA library was constructed using the SMART™ cDNA Library Construction Kit (Clontech, UK). This product was chosen for a variety of reasons. Firstly, the cDNA library can be generated from as little as 50 ng mRNA. This is a useful modification from other cDNA library synthesis kits as, in this project, the number of mites available was limited, and so the resulting yield of mRNA was also limited. Secondly, the Clontech kit utilises technology that results in libraries with a higher proportion of full-length cDNAs when compared to more traditional cDNA library preparations. Finally, the cDNA inserts are directionally cloned, have blue/white colour selection, and can easily be excised from the phagemid to the plasmid in a one-step process. This means that the inserts can be sequenced and cloned into expression vectors with relative ease.

The primary *P. ovis* cDNA library had a titre of 1.2×10^6 pfu/ml with a high percentage (89%) of recombinant phage. Insert analysis (Figure 4.2) showed that the majority of recombinant phage contained inserts larger than 500 bp, confirming the quality of the library.

Western blots were carried out prior to immunoscreening to determine the protein profile recognised by the sheep anti-*P. ovis* S1/S2 sera (Figure 4.3). A large number of bands were recognised by sera from both the S1 and S2 immunised animals. The negative control sera recognised three bands at approx. 180 kDa, 50 kDa and 25 kDa (Figure 4.3, lane 3). Further analysis (Figure 4.3, lane 2) indicated that this was due to cross-reactivity between the mite antigen and the secondary antibody (donkey anti-sheep IgG), as the bands were recognised in the absence of primary antibody. Despite extensive washing prior to mite extract preparation, the mite extracts used are likely to contain proteins of ovine host origin. For example, the proteins may include ovine IgG, as ingestion of this has previously been demonstrated (Pettit et al., 2000). Under non-reducing conditions ovine IgG migrates in SDS-PAGE at approx. 180 kDa, and ovine albumin migrates at approx. 50 kDa (Pettit et al., 2000). These sizes correspond to the bands recognised in the mite antigen by the secondary antibody and may therefore offer an explanation for the presence of the cross-reactive bands.

The immunoscreen identified 48 positives of which six were recognised by sera from animals strongly protected from mite challenge but not from animals weakly protected, suggesting that they may be important in the protective immune response. Five of the positives had homology to catchin, a myosin rod-like protein, which is found in the catch muscles of molluscs. Catch muscles are thought to perform a unique function whereby they can maintain high levels of tension with a minimum requirement of energy. These proteins are derived by alternative splicing of the myosin heavy chain gene (Yamada, Yoshio, Oiwa et al., 2000). The N-terminal of the catchin proteins are highly conserved and unique to catch muscles, whereas the rest of the molecule is highly similar to the C-terminal of the myosin heavy chain gene (Yamada et al., 2000). In the present study, homology to catchin was slightly more pronounced at the N-terminal end of the sequences analysed, with myosin homology more pronounced at the C-terminal. Myosin-based proteins have been identified as putative vaccine candidates in other organisms. For example, myosin is regarded as a valid vaccine candidate against *Schistosoma mansoni* infection. The rIrV-5 antigen derived from *S. mansoni*, is a 62 kDa fragment of a 200 kDa myosin II heavy chain molecule, which is expressed on the surface of newly transformed schistosomulum. Vaccination with this recombinant antigen resulted in 75% protection in mice (Soisson, Masterson, Tom et al., 1992), but in baboons, vaccination with rIrV-5 resulted in variable protection. Protection was correlated with the titre of anti-IrV-5 antibodies suggesting protection was antibody mediated (Soisson, Reid, Farah et al., 1993). Studies with the IrV-5 homologue, Sj62, from the Asian schistosome, *S. japonicum*, however have not been so successful. No protective effects were observed when either mice, rats or pigs were vaccinated with the recombinant antigen (Zhang, Taylor, & Bickle, 1998; Bickle, Bøgh, Johansen et al., 2001). This may be because the Sj62 antigen is not found on the surface of newly transformed schistosomula (Zhang, Taylor, & Bickle, 1998).

Further work is required to define the extent of the correlation between the level of immunity induced by vaccination with *P. ovis* S1 and S2 and the specificity of the antibody response. This could be achieved by using an ELISA directed against the recombinant protein product of the plaques isolated in this study and seeking correlates of protection with antibody titre, antibody being considered the effector of

protection (Smith et al, 2002). On a cautionary note, myosin is often encountered in immunoscreens using antisera raised against parasite extracts because it is thought to be highly immunogenic (D.P. Knox; Personal Communication). If a correlation between protection and myosin antibody titres was observed, it would be necessary to express the myosin-like fragments in a suitable expression vector and conduct vaccination trials to determine if the myosin-like proteins had any protective effects.

The screening of the *P. ovis* cDNA library with sera from strongly and weakly protected individuals may not have identified all the potential differentially recognised proteins. Analysis of the *P. ovis* proteins recognised on a 2D gel by western blotting with the highly or weakly protected sera may identify other proteins of interest.

Other studies carried out at Moredun Research Institute have identified three high molecular weight allergens (J. Huntley; Personal Communication). These allergens were identified as bands on Western blots probed with monoclonal mouse anti-sheep IgE antibody and N-terminal amino acid sequence data suggests that they may have homology to myosin, paramyosin and vitellogenin. There is the possibility that the myosin identified here is a homologue of those identified by Dr. Huntley, however further studies are required to confirm this theory.

Despite showing no homology to any protein sequences in the public databases, the relevance of remaining positive, Po41, to protection cannot be discounted.

The cDNA library could be screened with a monoclonal mouse anti-sheep IgE antibody, 2F1, which has recently been obtained at Moredun (J. Huntley; Personal Communication). Screening the cDNA library with this monoclonal mouse anti-sheep IgE antibody may identify proteins that are associated with the hypersensitivity response, and therefore, involved in the pathogenesis of the disease. The cDNA library may also be useful in the identification and production of recombinant forms of the three high molecular weight allergens. For example, the library could be screened with antisera to the native proteins and if cDNA clones were identified this would facilitate the production of recombinant allergen which could then be used in further trials e.g. ELISA or skin test assays. Another method to extract useful data from the cDNA library would be to conduct an expressed

sequence tag (EST) analysis. As the results from the cDNA immunoscreen did not highlight as many proteins of interest as was first expected, an EST analysis has been conducted and is presented in Chapter 5.

CHAPTER 5: EST ANALYSIS

5.1 INTRODUCTION

A brief introduction will be given here, as the main introduction for this topic is discussed in Chapter 4: cDNA Library Synthesis and Immunoscreening.

As discussed earlier (chapter 4) the mite antigens that evoke the immune response in sheep remain largely undefined. Biochemical analysis suggests that there are many protease enzymes present in *P. ovis* mites, some of which may induce the immediate hypersensitivity response observed (Nisbet & Billingsley, 1999b; 2000; Kenyon & Knox, 2002). A comparatively new approach that might reveal new proteins of interest, is to conduct an expressed sequence tag (EST) analysis. In this approach, relatively short sequences derived from randomly selected cDNAs can be used to define the genes expressed by an organism (Adams, Kelley, Gocayne et al., 1991; Waterston, Martin, Craxton et al., 1992; Blaxter, Raghavan, Ghosh et al., 1996; Blaxter, Aslett, Guiliano et al., 1999; Daub et al., 2000). The method involves single pass sequencing of randomly selected recombinant clones from a cDNA library followed by database searches to identify the nature of the proteins encoded. This method has been used with success in the identification of genes from a variety of parasites including the cattle tick, *Boophilus microplus* (Crampton, Miller, Baxter et al., 1998), the ovine gastrointestinal nematode, *Haemonchus contortus* (Hoekstra, Visser, Otsen et al., 2000), the filarial nematodes, *Brugia malayi* (Blaxter et al., 1996), *Onchocerca volvulus* (Lizotte-Waniewski, Tawe, Guiliano et al., 2000) and the human hookworm, *Necator americanus* (Daub et al., 2000). EST analysis can be used to identify proteins for novel vaccine candidates, drug targets or chemotherapy, as described for *O. volvulus* (Lizotte-Waniewski et al., 2000), to compare the genes expressed by different life stages of organisms or to determine when certain genes are switched on or off.

The present study was initiated with the aim of rapidly identifying further potential allergens and other proteins of importance to the pathogenesis of *P. ovis* infestation using EST analysis.

5.2 RESULTS

5.2.1 The EST Dataset

Of 501 clones (>500 bp in size) successfully sequenced, 268 (53.5%) showed significant ($e > 10^{-8}$) homology to existing database sequences. 251 could be grouped into 52 distinct clusters of >1 EST with 30 of these having significant identity to existing sequences. Of the 250 sequences that appeared on only one occasion (singletons), 139 showed significant homology to existing sequences. The whole EST dataset can be accessed in the NCBI database at <http://www.ncbi.nlm.nih.gov/dbEST>, accession numbers BQ834597 to BQ835080 and can also be viewed on the internet at <http://nema.cap.ed.ac.uk/Chelicerates/ChelDB.php>. For ease of reference, some of the ESTs with potential relevance to the disease process or of general biological interest are listed in Table 5.1.

5.2.2 ESTs Selected for Further Analysis

The focus of this study was to identify ESTs that encode proteins of potential importance in the pathogenesis of the sheep scab mite infestation. Therefore, ESTs were selected for further analysis based on their homology to known allergens, proteases or antioxidant enzymes (Table 5.1). These include ESTs that encode proteins with homology to confirmed allergens in house dust mites (*Dermatophagoides* spp.), storage mites (*Lepidoglyphus* spp.) and tropical mites (*Blomia* spp.). In addition, the dataset includes ESTs that encode several proteases and a range of antioxidant enzymes.

Sequence designation cluster/accession no	No. ESTs	Gene name	Putative function	Homologue in	E- value	ORF	% Homology (Blast X)	
							Identity	Similarity
<i>Allergens</i>								
007/BQ834604	19	<i>Pso o</i> II	Group II allergen	<i>P. ovis</i>	1.2 e ⁻⁷²	160	99	99
016/BQ834615	9	Pso Der F A	Secreted cysteine protease	<i>Dermatophagoides farinae</i>	2.1 e ⁻⁷³	231	69	79
011/BQ834608	4	Pso-GST	Glutathione S-transferase	<i>Drosophila melanogaster</i>	9.8 e ⁻²¹	141	45	65
022/BQ834621	4	Pso-FABP	Fatty acid binding protein	<i>Blomia tropicalis</i>	8.8 e ⁻²⁰	134	35	53
130/BQ834778	3	Pso-Gp7	Unknown, group 7 allergen	<i>Lepidoglyphus destructor</i>	1.2 e ⁻²²	178	35	57
275/BQ834975	2	Pso Der p V	Der p V IgE binding allergen	<i>D. pteronyssinus</i>	8.0 e ⁻³⁵	154	40	64
BQ834874	1	Pso Tropo	Tropomyosin	<i>D. farinae</i>	7.6 e ⁻⁹⁴	212	98	98
BQ834786	1	Pso Paramy	Paramyosin	<i>Sarcoptes scabiei</i>	1.5 e ⁻⁵⁸	172	90	93
BQ835043	1	Pso-HSPI	Heat shock protein	<i>Caenorhabditis elegans</i>	9.2 e ⁻¹³	149	41	58
<i>Cysteine proteases</i>								
016/BQ834615	9	Pso Der F A	Secreted cysteine protease	<i>D. farinae</i>	2.1 e ⁻⁷³	231	69	79
BQ834710	1	Pso CathB1	Cathepsin B-like cysteine protease	<i>Bombyx mori</i> (silk moth)	1.5 e ⁻⁴⁹	204	48	63
BQ834913	1	Pso CathB2	Cathepsin B-like cysteine protease	<i>C. elegans</i>	6.1 e ⁻¹⁶	57	72	84
BQ834906	1	Pso CathL1	Cathepsin L-like cysteine protease	<i>Sitophilus zeamais</i> (maize weevil)	1.3 e ⁻⁸²	239	65	76
<i>Free-radical scavenging enzymes</i>								
011/BQ834608	4	Pso-GST	Glutathione S-transferase	<i>D. melanogaster</i>	9.8 e ⁻²¹	141	45	65
BQ834873	1	Pso-Tpx1	Thioredoxin peroxidase	<i>Globodera rostochiensis</i>	9.4 e ⁻⁷³	234	75	87
BQ834914	1	Pso-Tpx2	Thioredoxin peroxidase	<i>Homo sapiens</i>	8.8 e ⁻⁶⁸	210	68	85
BQ834951	1	Pso-Tpx3	Thioredoxin peroxidase	<i>D. melanogaster</i>	5.9 e ⁻⁶²	196	85	87
BQ834990	1	Pso-SOD	Superoxide dismutase	<i>Oryza sativa</i> (rice)	1.2 e ⁻⁴⁹	149	70	79

Sequence designation cluster/accession no	No. ESTs	Gene name	Putative function	Homologue in	E- value	ORF	% Homology (Blast X)	
							Identity	Similarity
Others of interest								
004/BQ834600	19	Pso-LIM	LIM domain containing protein	<i>C. elegans</i>	5.5 e ⁻²⁷	106	59	73
087/BQ834722	2	Pso-cystatI	Cysteine protease inhibitor	<i>Bos taurus</i>	6.3 e ⁻¹⁰	155	42	58
222/BQ834901	2	Pso-PKC	Protein kinase C	<i>Aplysia californica</i>	2.3 e ⁻³⁷	173	48	65
327/BQ835048	2	Pso-cytoC	Cytochrome C oxidase	<i>Homo sapiens</i>	1.3 e ⁻¹⁶	218	26	51
BQ834616	1	Pso-PDI	Protein disulphide isomerase	<i>Mus musculus</i>	2.2 e ⁻³⁰	213	62	76
BQ834761	1	Pso-FSP	Fungal stress protein	<i>Rhizopus nigricans</i>	4.0 e ⁻⁴⁰	238	43	64
BQ834767	1	Pso-ADH	adenosylhomocysteinease	<i>C. elegans</i>	2.8 e ⁻⁷¹	188	77	85
BQ834801	1	Pso- proteosome	Proteasome subunit beta type 4 precursor	<i>Xenopus laevis</i>	1.8 e ⁻⁴⁶	238	54	73
BQ834896	1	Pso-IgE-BF	IgE binding factor	<i>Homo sapiens</i>	3.8 e ⁻¹⁰	251	33	47
BQ834927	1	Pso-CutI	Rigid cuticular protein	<i>Araneus diadematus</i> (spider)	2.8 e ⁻⁰⁹	165	50	55
BQ834991	1	Pso-ALG2	Programmed cell death protein	<i>Mus musculus</i>	2.3 e ⁻⁵³	184	57	77
BQ835077	1	Pso- ALADH1	Haem biosynthesis enzyme	Fission yeast	8.1 e ⁻³⁷	100	38	54

Ribosome-associated proteins N=36

Table 5.1. Summary of the EST dataset showing those ESTs encoding proteins with homology to allergens, proteases, free-radical scavenging enzymes and others of general interest. For the clusters the accession number of the first EST in the cluster is shown. All other ESTs in the cluster can be viewed at <http://nema.cap.ed.ac.uk/Chelicerates/ChelIDB.php>.

Allergens

In total, 44 ESTs were identified that showed significant homology to allergens of other organisms. Of these, nineteen (4 % of the total, derived from cluster 007) showed homology to genes encoding a recently identified *P. ovis* allergen, *Pso o* II (Table 5.1, Temeyer, Soileau, & Pruett, 2002). This allergen is closely related to the group 2 allergens from the house dust mites (*Dermatophagoides* spp.). Nine ESTs (derived from cluster 016) encoded a protein that shared homology with the group 1 allergens, which are cysteine proteases e.g. *Der f*1 (Table 5.1).

Four ESTs (cluster 022) encoded a protein that has homology to a fatty acid binding protein (FABP) allergen from the tropical mite, *Blomia tropicalis*. The FABPs are also known as the group 13 mite allergens, and an alignment of these sequences is shown in Figure 5.1. The *Pso*-FABP sequence is 36% identical to the *Blo t* 13 allergen sequence, and is 37% identical to the *Lep d* 13 allergen. An N-glycosylation site is present at residues 122-125. In addition, 6 of 7 conserved glycine residues and a conserved arginine residue are present in the *P. ovis* FABP sequence (Sacchettini, Banaszak, & Gordon, 1990). A cluster comprising 2 ESTs had homology to the group 5 house dust mite allergens (Figure 5.2). A signal peptide cleavage site is present between amino acids 18 and 19. A further cluster comprising 3 ESTs (cluster 130) showed closest homology to a group 7 mite allergen, *Lep d* 7, from the storage mite, *Lepidoglyphus destructor* (Figure 5.3). In this sequence 4 N-glycosylation sites are present at residues 54-57, 88-91, 92-95 and 121-124, and a predicted signal peptide cleavage site is present at amino acid 22. Three other putative allergens were identified, each represented by a single EST, and they encoded tropomyosin, paramyosin and a heat shock protein respectively (Table 5.1).

```

      *          20      *          40      *
Blo t 13 : MP-IEGKYKLEKSDNFDKFLDELGVGFMVKTAAKTLKETLEVDVQGDITYV : 49
Lep d 13 : MANIAGQYKLDKSENFQFLDKLGVGFLVKTAAKTVKPTLEVAVDGDITYI : 50
Smansoni : MSSFLGKWKLSSESHNFDAVMSKLGVSWATRQIGNIVTPTVTFTMDGDKMT : 50
Povis    : MA-VEGKYQMESSEHFEEFIKETGFTVDLSNVDRSKTSTLEVHRDGDVVFH : 49

      60      *          * 80      *          100
Blo t 13 : FRSLSTFKNTEIKFKLGEEFEEDRADGKRVKTVVNKEGDNKFIQTQ--YG : 97
Lep d 13 : FRSLSTFKNTEIKFKLGEEFEEDRADGKRVKTVIVKDGDNKQVQTQ--YG : 98
Smansoni : MLTSTFKNLSCTFKFGEEFDEKTS DGRNVKSVVEKNSESKLTQTQ--VD : 98
Povis    : LKSDMAGVGHEIKFKLGEEFEDEMNGHKFNVNMDGEHKMVQTKTSVD : 98

      ↓      *          120
Blo t 13 : DK-EVKIVRDFQGGDVVVTASVGDVTSVRITYKRI- : 130
Lep t 13 : DK-EVKVVRDFQGGDVVVTASVGDVTSVRIPYKRA- : 131
Smansoni : PKNTTVIVREVDGDTMKTIVTVGDVTAIRNYKRLS : 133
Povis    : GGKVIHVREFTDAGCTVKTIVNNVTWTRVYKRV- : 132

```

Figure 5.1 Alignment of the group 13 allergens, putative fatty acid-binding proteins. Residues shaded in black are 100% identical, residues shaded in grey are 80% similar. -, gap inserted to improve alignment, *, conserved glycine residues, ↓ conserved arginine residues (Sacchettini, Banaszak, & Gordon, 1990). The sequences used and their GenBank accession numbers are as follows: *Blo t 13*, *Blomia tropicalis*, Q17284; *Lep d 13*, *Lepidoglyphus destructor*, Q9U5P1; *S. mansoni*, *Schistosoma mansoni* fatty acid binding protein, P29498; *Povis*, Pso-FABP, BQ834621.

```

      20          40
Lep d 5 : -----DD--FRNEFDRLLIHMTEEQFAKLEQ : 24
Blo t 5 : MKFATVLIACFAASVLAQEHKPKKDD--FRNEFDHLLIEQANHAIEKGEH : 48
Der p 5 : MKFITAFFVATLA-VMTVSGEDKKHD--YQNEFDLLMERIHEQIKKGEL : 47
Povis gp5: MKFITALCALAMAITVSGMVICKNDEDKWHNEFDRLLIMEEIEDKYDELER : 50

      60      80      100
Lep d 5 : ALAHLSHOVTELEKSKSKELKAQILREISIGLDFIDSAGKHFERELKREAD : 74
Blo t 5 : QLLYLQHOLDENENKSKELQEKTIREDLVVCAMIEGAQCALERELKRTD : 98
Der p 5 : ALFYLQEQINHFEKPTKEMKDKIVAEMDTIIAMIDGVRGVLDRLMQRKD : 97
Povis gp5: GLLVLSMNYKELLKTHDKELKEKNST----- : 76

      120
Lep d 5 : LNLAEKFNFEALSTGAVLHKDITATATKVKATETK : 110
Blo t 5 : LNILERFNYYEAQTLISKILLKDLKETEQKVVDIQTQ : 134
Der p 5 : LDIFEQYNLEMAKKSGDILERDLKKEEARVKKIEV- : 132
Povis gp5: ----- : -

```

Figure 5.2 Alignment of the group 5 allergens. Residues shaded in black are 100% identical those shaded in grey are 80% similar. -, gap inserted to improve alignment. The sequences used and the GenBank accession numbers are: *Lep d 5*, *Lepidoglyphus destructor*, Q9U5P2; *Blo t 5*, *Blomia tropicalis*, Q96870; *Der p 5*, *Dermatophagoides pteronyssinus*, P14004; *Povis_gp5*, Pso Der p V from EST analysis, BQ834975.


```

                20                      40
Pso Gp7 : --MKFFTYLIAFIAIYSGASVHGDDKTDQVNTFIDQILQSVLKMRPLDEL : 48
Derp7   : -MKLLLLIAAAAFVAVSADPIHYDKITEEINKAVDEAVAAIEKSETFDPM : 49
Derf7   : -MKFLLIAAVAFVAVSADPIHYDKITEEINKAIDDAIAAIEQSETIDPM : 49
Lepd7   : MQYLAIIVIVLAGLSAAAHKPAYYDDNMANQMVDQIVKSLTTKKELDPF : 50

                60                      80                      100
Pso Gp7 : KLPNNQTIDRKIGVITPLHIEFLHMQNTIEIYGLGHIHRNGNASLNSTGGQF : 98
Derp7   : KVPDHSDKFERHIGIIDLKGEEDMRNTQVRGLKQMKRVGDANVKSEDEGVV : 99
Derf7   : KVPDHADKFERHVGIVDFKGEIAMRNIEARGLKQMKRQGDANVKGEEGIV : 99
Lepd7   : KIEQTKVPIDKKIGLTHIKGSATIKNAVITGLSHISRGRDAKIDTDGGAFF : 100

                120                      140
Pso Gp7 : DLKFRLNDDHVIARSDDLINFNN-HSYPNLKAIEDTIGDIDVKLEIAVLS- : 146
Derp7   : KAHILVGVHDDVVSMEYDLAYKLGDLHPNTHVISDIQDFVVELSIEVSEE : 149
Derf7   : KAHILIGVHDDIVSMEYDLAYKLGDLHPTTHVISDIQDFVVALSIEISDE : 149
Lepd7   : AATIKLGDKNIRIKTDLHLDLGK-IIEPNLKFEHGIDIDMKIKKLDAAE : 149

                160                      180                      200
Pso Gp7 : GKFDIKSFEVDELKNCKVYMHGLKG----- : 171
Derp7   : GNMTLTSFEVRQFANVNVNHIGGLSILDPFAVLSQVLTAFIQDTRVFAEMT : 199
Derf7   : GNITMTSFEVRQFANVNVNHIGGLSILDPFVGLSDVLTAFIQDTRVKEMT : 199
Lepd7   : GKPSLDQFEIDEFEQVELFTIHGLGPLDPLVDVIADSFVKYENPQARKLVT : 199

Pso Gp7 : ----- : -
Derp7   : KVLAPAFKKELERNNQ- : 215
Derf7   : KVLAPAFKRELEKN--- : 213
Lepd7   : DMLKPILVEEIKKLKLN : 216

```

Figure 5.3 Alignment of the group 7 allergens. Residues shaded in black are 100% identical those shaded in grey are 80% similar. -, gap inserted to improve alignment. Sequences used and their GenBank accession numbers were: Pso Gp7, *P. ovis* from the EST analysis, BQ834778; Lep d 7; *Lepidoglyphus destructor*, Q9U1G2; Der p 7; *Dermatophagoides pteronyssinus*, P49273; Der f 7; *D. farinae*, Q26456.

Proteases

This study also identified four distinct types of cysteine protease. Two types of cathepsin B protease were identified, Pso CathB1 and Pso CathB2, and one cathepsin L cysteine protease, Pso CathL1 (Table 5.1). A cysteine protease (Pso Der f A) was also identified with homology to the major house dust mite group 1 allergen. A detailed analysis of these sequences is presented in Chapter 6.

Antioxidant enzymes

Three types of antioxidant enzyme were identified; glutathione S-transferase (Pso-GST), superoxide dismutase (Pso-SOD), and thioredoxin peroxidase (Tpx). Pso-SOD was homologous to the copper/zinc (Cu/Zn) superoxide dismutases.

The proteins encoded by three ESTs with homology to thioredoxin peroxidase were aligned (Figure 5.4). This revealed that there are two types of thioredoxin peroxidase present in the mites. Pso-Tpx1 and Pso-Tpx3 were almost identical (78% identical) to each other and differing from Pso-Tpx2 (50% identity with Pso-Tpx1 and Pso-Tpx3). These sequences were aligned with thioredoxin peroxidases from other organisms (Figure 5.5) and this analysis identified 71 identical residues in 187 (Tpx 2 as reference) with a conserved FVCP motif at positions 51-54 and a further conserved Cys residue at position 174 (shown by asterisks) both of which are characteristic of 2-Cys thioredoxin peroxidases.

A cluster of four ESTs encoded a protein with homology to glutathione S-transferase (Pso-GST). A GST has recently been characterised from *P. ovis* (*P. ovis* GST1, (Lee et al., 2002)). The GST identified here, *P. ovis* GST2, shows limited homology to *P. ovis* GST1 (9 % identity and 22 % similarity over 129 amino acids) but showed much closer homology (45% identity and 65% similarity) to a GST identified from *D. melanogaster*. The sequence identified by Lee et al (2002) was described as a mu-class GST. Phylogenetic analyses (Figure 5.6, Lee et al., 2002) suggested that *P. ovis* GST2 is a member of the theta class of GST molecules.

```

                                20                                40
Povis tpx1: MAQS--GPRVQCPAPQFKGEAVVNGQFKETSLADYKGYVVLFFYPMDFT : 48
Povis tpx3: MAQS--GPRVQCPAPQFKGEAVVNGQFKETSLADYKGYVVLFFYPMDFT : 48
Povis tpx2: MPSRSMsAIVQRQAPYFAATAVVDKQFKEVKLTDFQGKYLVLFFYPMDFT : 50

                                60                                80                                100
Povis tpx1: FVCPTETIAFSERADEFKMNCEVLAACSTDSKFTHLAWINTPRKEGGLGE : 98
Povis tpx3: FVCPTETIAFSERADEFKMNCEVLAACSTDSKFTHLAWINTPRKEGGLGE : 98
Povis tpx2: FVCPTETIAFNDRLKEFHDLDTAVVAVSVDSHFSLAWCNTPRKQGGLGD : 100

                                120                                140
Povis tpx1: MNTPHIAADKNGKISQDYGVILEDG-VPLRGLFIIDGNOILRQITINDLPV : 147
Povis tpx3: MNTPHIAADKNGKISQDYGVILEDG-VPLRGLFIIDGNOILRQITINDLPV : 147
Povis tpx2: MKMPIISDLTKKISEDYGVLPDAGIALRGLFIIDANGIVRQITINDLPV : 150

                                160                                180                                200
Povis tpx1: GRNVDETIRLVCAFOYTDTHGEVCPANLEPGSDTIKPAQTGAKNFSPKTI : 197
Povis tpx3: GRKCDDETIRLVCAISITLII-----HCGK----- : 171
Povis tpx2: GRSVDETIRLRIKAFQYTDKNGEVCPAN-----WQPKETDNQI- : 187

Povis tpx1: GVIWNDEKK : 206
Povis tpx3: ----- : -
Povis tpx2: ----- : -

```

Figure 5.4 Alignment of the deduced protein sequences of the three thioredoxin peroxidases identified from the EST dataset.

```

                                20                                40
Povis tpx2: MPSRMSAIVQRQAPYFAATAVVDKQFKFVKLTDFQCKYLALFFYPPLDFT : 50
Povis tpx1: --MAQSGPRVQKEAPQFKGEAVVNGQFKETSLADYKGYVVLFFYPMDFT : 48
A suum : ----MSKAMIGKPAPEFTATAVVDGDFKSLISLSDYKGYVVLFFYPMDFT : 46
O volvulus: MTLAGSKAFIGQAPNFKTTAVVNGDFKETSLNQFKGYVVLFFYPPLDFT : 50
A aegypti : ----MPVPDLQKEAPKFSGTAVVNGAFKETKLEDYAGKYLALFFYPPLDFT : 46
F hepatica: ----MLQP--NMEAPNFSGQAVVGKEFETISLSDYKGWVILAFYPLDFT : 44

                                *                                60                                80                                100
Povis tpx2: FVCPTEIIAFNDRLKEFHDLDETVVAVSVDSHFSLAWCNTPRKQGGLGD : 100
Povis tpx1: FVCPTEIIAFSERADEFRKMNCVEVIACSTDSKETHLAWINTPRKEGGLGE : 98
A suum : FVCPTEIIAFSEHVGEFKKLGVVLAASTDSQFSLAWINTPRKQGGLGE : 96
O volvulus: FVCPTEIIAFSDRISEFKKLDVAVMACSTDSHFSLAWVNTTEKWVGLGQ : 100
A aegypti : FVCPTEIIAFSDRVEEFKIGCSVIGVSTDSHFTHLAWINTPRKQGGLGE : 96
F hepatica: FVCPTEIIAISDQMEQFAQRNCAVIFCSTDSVYSHLQWTKMDRKVGGIGQ : 94

                                120                                140
Povis tpx2: MKMPIISDLTKKISEDYGVLIPDAGIALRGLFIIDANGIVRQITINDLPV : 150
Povis tpx1: MNIPLLADKNGKLSQDYGVYLED-GVPLRGLFIIDGNQITRQITINDLPV : 147
A suum : MKIPIISDNNHQLSRDYGVLKEDDGIAYRGLFIIDPKGITRQITVNDLPV : 146
O volvulus: MNIPILADTNHAI SKAYGVLEKDEGIAYRGLFIIDSKGITRQITVNDLPV : 150
A aegypti : LRIPLLADKSMKLSRDYGVLQEESEGVFPFRGLFVIDGQNLRQITVNDLPV : 146
F hepatica: LNFPLLADKNMSVSRAFGVLDDEEQGNTYRGNFLIDPKGVLTRQITVNDLPV : 144

                                160                                *                                180                                200
Povis tpx2: GRSVDETLRLIKAFOYTDKHGEVCPANWQP-----KETDNQI---- : 187
Povis tpx1: GRNVDETIRLVQAFQYTDIHGEVCPANLEPGSDTIKPAQTGAQNFSPKTI : 197
A suum : GRSVDETLRLVQAFQFVDKHGEVCPAGWTPGADTIKPGVKESKAYFEKH- : 195
O volvulus: GRSVDETLRLIQAFQFVDNHGEVCPANWQPGSETIKPEVKESKEYFGKH- : 199
A aegypti : GRSVDETLRLVQAFQFTDEHGEVCPANWKPGSKTMVADPQKSKEYFNAAN : 196
F hepatica: GRSVEEALRLLDAFIFHEEHGEVCPANWKPKSKTIVPTPDGSKAYFSSAN : 194

Povis tpx2: ----- : -
Povis tpx1: GVIWNDEKK : 206
A suum : ----- : -
O volvulus: ----- : -
A aegypti : ----- : -
F hepatica: ----- : -

```

Figure 5.5 Alignment of the thioredoxin peroxidase protein sequences. Residues shaded in black are 100% identical those shaded in grey are 80% similar. -, gaps inserted to improve alignment. *, conserved residues. Sequences used and their GenBank accession numbers are: *Ascaris suum*, Q9NL98; *Onchocerca volvulus*, O44366; *Aedes aegypti*, Q8WSF6; *Fasciola hepatica*, P91883. *Psoroptes ovis* Tpx 1 and 2 were identified from the EST analysis.

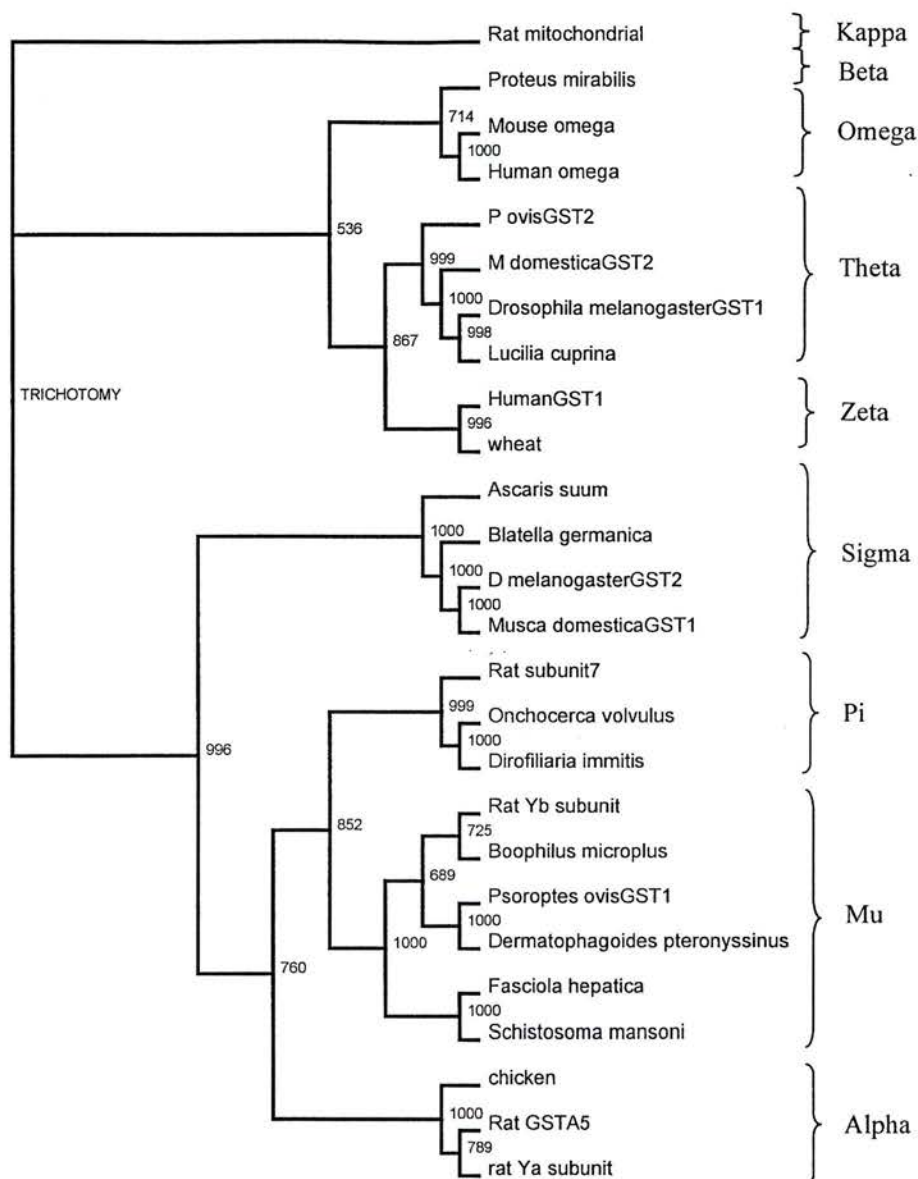


Figure 5.6 Phylogenetic analysis of GST protein sequences. Annotation to the right of the diagram denotes the class of GST. The bootstrap values are indicated at the nodes of the tree. The GenBank accession numbers are as follows: Rat mitochondrial, P24473; *Proteus mirabilis*, P15214; Mouse omega, U80819; Human omega, AF212303; *P. ovis* GST2, GST homologue identified in the EST analysis, BQ834608; *Musca domestica* GST2, P46431; *Drosophila melanogaster* GST1, P20432; *Lucilia cuprina*, P42860; Human GST1, AAB96392; Wheat, AAD09190; *Ascaris suum*, CAA53218; *Blatella germanica*, O18598; *D. melanogaster* GST2, P41043; *M. domestica* GST1, AAA03434; Rat subunit 7, P04906; *Onchocerca volvulus*, AAA53575; *Dirofilaria immitis*, P46426; Rat Yb subunit, P04905; *Boophilus microplus*, AAD15991; *P. ovis* GST1, AAF19264; *Dermatophagoides pteronyssinus*, P46419; *Fasciola hepatica*, P56598; *Schistosoma mansoni*, P35661; chicken, Q08393; Rat GSTA5, A54858; Rat Ya subunit, P00502.

Abundant Novel ESTs

In this dataset 233 (46.5 %) of the ESTs did not have significant hits in the sequence databases, so were classed as novel ESTs. These novel ESTs comprised many singletons and 23 clusters. Those clusters that were highly abundant in the dataset i.e. comprising 8 or more ESTs, were analysed for the presence of signal peptides using the signal peptide search option on the SMART website (<http://smart.embl-heidelberg.de>) and the pattern search programme on the Protein Information Resource (PIR) website (<http://pir.georgetown.edu/pirwww/search/patmatch.html>). The sequences selected were as follows: cluster 006 (accession number BQ834602, 15 ESTs), cluster 026 (accession number BQ834628, 9 ESTs), cluster 028 (accession number BQ834633, 17 ESTs), cluster 058 (accession number BQ834680, 8 ESTs), cluster 094 (accession number BQ834730, 13 ESTs) and cluster 102 (accession number BQ834739, 10 ESTs). Four of these sequences (clusters 006, 026, 094, and 102) were found to contain a leader signal peptide.

5.3 DISCUSSION

This study was initiated with the aim of rapidly identifying further putative allergens and other proteins of potential importance to lesion formation during *P. ovis* infestation. The success of this approach was illustrated by the discovery of 44 ESTs (9% of the total) with homology to genes encoding known allergens previously identified in house dust, storage and forage mite species (Table 5.1). These mite species contain several allergens with interspecies similarities that lead to immunological cross-reactivity. The allergens can be loosely divided into two categories, namely those with or without enzyme activity (Stewart, 1995). The allergens with enzyme activity are mostly hydrolases associated with digestion, derived from the gut and excreted from the mite in faecal pellets, which contain a range of enzymes and other proteins (Stewart, 1995). Proteases may play a role in initiation of the lesion and therefore contribute to nutrient provision for the mite. The non-enzymatic allergens include heat shock proteins (Aki, Fujikawa, Wada et al., 1994), fatty acid binding proteins (Eriksson, Whitley, Johansson et al., 1999; Puerta et al., 1999) and structural proteins such as tropomyosin (Wittelman, Akkerdaas, van Leeuwen et al., 1994; Aki et al., 1995). A number of ESTs had homology to genes encoding free-radical scavenging enzymes, which may help the mite evade or modulate the host immune response.

Approx. 302 putative genes were identified from this EST analysis. Of these, only the *P. ovis* group 2 allergen homologue, *Pso o* II had been previously sequenced (Temeyer, Soileau, & Pruett, 2002). The group 2 allergen was the most abundant in the dataset, with 19 ESTs in the cluster. A single EST was also isolated that shared 94% identity with this sequence. The *Pso o* II gene was the most abundant in the dataset, but the small size of the dataset makes it impossible to say for certain that the gene is highly expressed in the mite. However, Daub et al. (2000) noted that early patterns of abundance in ESTs from the nematode *Brugia malayi* (Blaxter et al., 1996; Blaxter et al., 1999) were, in general, confirmed by more extensive sequencing. Sequence analysis of the group 2 allergens suggests that they are non-glycosylated proteins and contain leader peptide sequences that are cleaved to yield the mature protein. The mature protein is approx. 14kDa and contains three

disulphide bonds (Nishiyama, Yuuki, Takai et al., 1993), which are also present in the *P. ovis* homologue (Temeyer, Soileau, & Pruett, 2002). The allergenicity of this protein is dependent on a structurally intact immunoglobulin-like domain (Pomés & Chapman, 2001). These allergens are highly abundant in whole body extracts of the dust mites and are recognised by the majority of mite-allergic individuals (80 to 90%, Heymann, Chapman, Aalberse et al., 1989), but as yet have an undefined function in mite physiology. However, *Der p 2* is homologous to *esr16*, a gene that is expressed in moths around the time of moulting (Mueller, Benjamin, & Rule, 1998). This suggests that the group 2 allergens may have a role in mite development.

Nine ESTs encoded homologues of the house dust mite group 1 allergens, which are cysteine proteases (Chua et al., 1988). In *D. pteronyssinus*, *Der p 1*, the Pso Der F A homologue, is found in high concentrations in mite faeces and is present in the mite gut epithelium (Tovey, Chapman, & Platts-Mills, 1981; Tovey & Baldo, 1990). Whilst probably functioning as a digestive enzyme, it has been suggested that the proteolytic activity of *Der p 1* contributes to allergenicity (reviewed by Pomés & Chapman, 2001). A detailed sequence analysis has been carried out on the Pso Der F A sequence and it is presented in Chapter 6. Clearly, if the sheep scab mite homologue has similar allergenic properties to the house dust mite allergen, it could contribute significantly to the aetiology of the disease.

Four ESTs encoded a homologue of a mite group 13 allergen, *Blo t 13*, a fatty acid-binding protein (FABP) of the mite *Blomia tropicalis* (Figure 5.1, Caraballo, Puerta, Martin et al., 1997). The group 13 allergens have also been identified from the dust mites *Acarus siro* (Eriksson et al., 1999) and *Lepidoglyphus destructor* (Eriksson, Rasool, Huecas et al., 2002), and bind IgE in only 11% of sera from mite allergic individuals (Caraballo et al., 1997). FABPs are involved in general lipid metabolism, acting as intracellular transporters of hydrophobic metabolic intermediates and as carriers of lipids between membranes. In parasites they may also facilitate immune evasion, for example by binding immune mediators (McDermott, Cooper, & Kennedy, 1999). Of note, nematodes cannot synthesise complex lipids and must acquire them from the host (Barrett, 1981). Sheep scab mites live in a lipid rich environment on the surface of the skin and may be similarly dependent on the host for a supply of essential lipids. FABPs have been identified in

the midgut of larval *Manduca sexta*, the tobacco hornworm (Smith, Tsuchida, Hanneman et al., 1992), which is possibly indicative of a function in lipid uptake from the gut. The *P. ovis* sequence does not contain a putative signal peptide cleavage site indicating it is not secreted but contains a potential N-linked glycosylation site at amino acids 122-125. Six of seven glycine residues involved in the β -sheet orientation of FABPs (Sacchettini, Banaszak, & Gordon, 1990) are conserved in the *P. ovis* sequence (Figure 5.1). In addition, an arginine residue, which participates in electrostatic interaction with the carboxyl group of the fatty acid (Sacchettini, Banaszak, & Gordon, 1990), is conserved in all the sequences in the alignment (Figure 5.1).

A cluster comprising 3 ESTs showed closest homology to the allergen, *Lep d* 7, which is secreted from the storage mite *L. destructor* (Eriksson et al., 2002). This allergen is a close homologue of the group 7 allergens from *Dermatophagoides* spp (Figure 5.3). Although their biological function is undefined they are known to range from 26-31 kDa in size and bind IgE in sera from 50% of allergic people (Shen, Chua, Lin et al., 1993). The coding sequences for these proteins contain leader peptides indicative of secretion as well as a predicted N-linked glycosylation site (Shen et al., 1993). Antibody reactivity and deglycosylation studies have confirmed that these allergens are glycosylated *in vivo* (Shen, Chua, Lin et al., 1995). The glycosylation may explain the presence of different sized molecular species in mite extracts. The *P. ovis* sequence contains 4 potential N-linked glycosylation sites and a signal peptide cleavage site, which suggests that it may be secreted.

A further cluster of 2 ESTs showed 40% identity with *Der p* 5, an important allergen recognised by about 50% of mite-allergic individuals (Tovey, Johnson, Roche et al., 1989). *Der p* 5 homologues have been identified in *D. pteronyssinus* and *B. tropicalis*, although a homologue has not yet been identified in *D. farinae* mites. These allergens range in molecular weight from 14-17 kDa and their function is, as yet, unknown.

Three other putative allergens, namely tropomyosin, paramyosin and a heat shock protein, appeared as singletons in the EST dataset (Table 5.1). Tropomyosin is found in both vertebrates and invertebrates and is a structural protein involved in the regulation of calcium flux in muscles. Tropomyosin (group 10 allergen) has been

isolated from *D. farinae* (Aki et al., 1995) and binds IgE in about 80% of mite allergenic individuals. The native protein is approx. 37 kDa in size. Paramyosin is a high molecular weight allergen (approx. 98 kDa, group 11) and is recognised by greater than 80% of sera from mite allergic individuals (Tsai, Chao, Shen et al., 1998). Paramyosin is a core structural protein of the thick filaments of invertebrate muscle but is also a non-filamentous secreted protein (Matsumoto, Perry, Levine et al., 1988) and is likely to have other functions. Paramyosin has been shown to bind the C1 complement in other parasitic infections (Laclette, Shoemaker, Richter et al., 1992), which is possibly indicative of an immunomodulatory function. Homologues of the paramyosin allergen have been identified in the tropical mite *B. tropicalis* (Blo t 11) (Ramos, Nge, Wah et al., 2001), the mange mite, *Sarcoptes scabiei* (Mattsson, Ljunggren, & Bergström, 2001) and the fish parasite, *Anisakis simplex* (Pérez-Pérez, Fernandez-Caldas, Marañón et al., 2000), which causes human disease when accidentally ingested. It is possible that the group 11 allergens are involved in the hypersensitivity response, as they have been shown to bind IgE in Western blotting studies (Thomas & Smith, 1999; Ramos et al., 2001). Paramyosin is one of the primary candidate proteins for vaccine development against schistosomiasis (McManus, Wong, Zhou et al., 2002) and tropomyosin induces partial protective immunity in sheep against gastro-intestinal nematodes (Cobon, Kennedy, Wagland et al., 1989).

The heat shock protein identified in this study was homologous to the small heat shock protein family from *Caenorhabditis elegans*. Heat shock proteins (hsp) are a highly conserved family of proteins that are present in both pro- and eukaryotes, and are thought to have a role in protein folding. A cDNA encoding a protein with homology to the heat shock protein 70 (hsp70) family has been identified from the house dust mite, *D. farinae* (Aki et al., 1994).

The present study also identified three distinct cysteine protease encoding ESTs (Pso CathB1, Pso CathB2 and Pso CathL1), each represented once and not previously identified in other mite species (Table 5.1). In helminths, proteases of these types have been implicated, amongst other functions, in digestion (Knox, Redmond, & Jones, 1993; Rhoads & Fetterer, 1995; Fetterer & Rhoads, 1997a; Fetterer & Rhoads, 1997b) and in the penetration of tissue barriers (McKerrow,

1989; Knox, 1994; Tort et al., 1999). Some have been found in *in vitro* excretions/secretions, indicating a role in extracorporeal digestion. Hence, these proteases may also contribute to the disease syndrome and are worthy of further analysis. A detailed analysis of these proteases is presented in Chapter 6. Earlier work demonstrated the presence of aspartyl and metalloproteases in mite extracts, and it was suggested that these enzymes may be important in mite physiology or the aetiology of the disease (Chapter 3, Kenyon & Knox, 2002). However, no ESTs encoding these classes of proteases were identified here, which may simply reflect the small number of sequences sampled.

Three types of free-radical scavenging enzyme were identified in this study, namely superoxide dismutase (SOD), thioredoxin peroxidase and glutathione S-transferase (GST). Free radical scavenging enzymes neutralise potentially toxic free radicals such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^{\cdot}) (Henkle-Dührsen & Kampkötter, 2001). These toxic free radicals are produced by normal metabolism within cells, but are also produced by immune effector cells as part of a protective response (Callahan, Crouch, & James, 1988; Henkle-Dührsen & Kampkötter, 2001). The presence of free-radical scavenging enzymes in parasitic organisms has been linked with immunomodulation.

SOD catalyses the dismutation of the superoxide anion to hydrogen peroxide and oxygen (LoVerde, 1998). The SOD identified in this study is homologous to the copper/zinc form of the enzyme. It lacks a predicted signal peptide, indicating that it is the cytosolic form of the enzyme and is not secreted. It is therefore unlikely to be involved in the pathogenesis of the disease.

The peroxidoxins are a family of antioxidant enzymes which have only recently been identified and are characterised by having no cofactors (i.e. metals) (McGonigle, Dalton, & James, 1998). These enzymes primarily neutralise hydrogen peroxide thus preventing a build up of the toxic hydroxyl radical. Although thioredoxin peroxidases have been identified in many helminth parasites including *Onchocerca volvulus* (Lu, Egerton, Bianco et al., 1998), *Brugia malayi* (Ghosh, Eisinger, Raghavan et al., 1998), *Fasciola hepatica* (Salazar-Calderon, Martin-Alonso, Ruiz de Eguino et al., 2000) and *Schistosoma mansoni* (Kwatia, Botkin, & Williams, 2000), none have been identified to date from arthropod parasites. There

were three thioredoxin peroxidase genes identified in this analyses, two being essentially identical (Pso-Tpx1 and Pso-Tpx3) the third (Pso-Tpx2) being quite distinct (Figure 5.4). None of these sequences contained a predicted signal peptide cleavage site, indicating that they are not secreted. There are two main forms of thioredoxin peroxidases (Henkle-Dührsen & Kampkötter, 2001), the 1-cys and the 2-cys peroxiredoxins. Those identified in this study were of the 2-cys type. These enzymes are characterised by the presence of a conserved FVCP sequence around the first cysteine, and a second conserved residue in the C-terminal region, separated from the first by approx. 120 residues (Henkle-Dührsen & Kampkötter, 2001). These conserved cysteines form a disulphide bond between subunits resulting in homodimer formation (Hirotzu, Abe, Okada et al., 1999). These authors suggest that the cysteine disulphide bond is the catalyst in the reduction of hydrogen peroxide and may use thioredoxin as a source of reducing agents. The presence of multiple thioredoxin peroxidases suggests that these enzymes may be part of a multi-gene family and may be important in mite physiology.

GSTs are ubiquitous detoxifying enzymes which catalyse the conjugation of reduced glutathione with a number of electrophiles (LoVerde, 1998). In helminths, GSTs usually occur as several isoforms and play a central role in the parasite detoxification system including those targeting host-derived anti-parasite factors (Brophy & Pritchard, 1994). GSTs have also shown promise as vaccine targets (Spithill, Piedrafita, & Smooker, 1997). There are nine classes of cytosolic GST; alpha, beta, kappa, mu, omega, pi, sigma, theta and zeta. GSTs are grouped on the basis of a number of factors including substrate specificity, sequence homology and phylogenetic analyses. A GST homologue from *D. pteronyssinus* has been shown to be allergenic (O'Neill, Donovan, & Baldo, 1994) and, in fact, is representative of the group 8 mite allergens. These allergens are approx. 26 kDa in size, and the recombinant molecule is recognised by approx. 40% of mite allergenic individuals (O'Neill, Donovan, & Baldo, 1995). A GST homologue was recently identified in *P. ovis* (*P. ovis* GST1) and was classified with the mu-class GSTs (Lee et al., 2002). The present study revealed a variant, *P. ovis* GST2, which showed limited homology to *P. ovis* GST1, and phylogenetic analysis (Figure 5.6) indicates that this GST is a member of the theta class. These data indicate that *P. ovis* GSTs are derived from

multiple gene families. Potentially, the free radical scavenging enzymes identified may have a role in protecting mites from the host immune response. Reactive oxygen species are known to be released by immune effector cells as part of a protective immune response, so it is likely that the scab lesion could be a potent source of toxic free radicals. The GST, SOD and thioredoxin peroxidases identified in this analyses may therefore help the mite to prevent damage from reactive oxygen species that are released from the host.

In addition, one EST had homology to genes encoding a low affinity IgE receptor, or IgE binding factor (see Table 5.1, others of interest). These factors may be involved in IgE production and the differentiation of B-cells (Sutton & Gould, 1993) and may suggest that the mites may be capable of immunomodulation and alteration of the IgE response. Other proteins of potential interest identified included cystatin (a cysteine protease inhibitor) and a LIM domain containing protein (Freyd, Kim, & Horvitz, 1990), which may be involved in myogenesis.

A large proportion (47.5%) of the ESTs sequenced did not have any significant hits during the database searches, and were therefore classed as novel sequences. Of the 23 clusters that encoded novel ESTs, six clusters comprised 8 or more ESTs suggesting relatively abundant protein products. Of the six clusters analysed, four were found to have signal peptides, suggesting that they could be secreted by the mites and therefore play a role in the pathogenesis of the disease.

To summarise, this analysis identified several ESTs encoding putative proteins, including allergens, proteases and free radical scavenging enzymes that are likely to contribute to lesion formation and disease pathogenesis. Future work is required to determine if the putative allergens that have been identified in this study, are in fact allergens. This can be determined by expressing each of the clones of interest in a suitable expression vector, such as pGEX, then carrying out ELISA to determine the capacity for IgE recognition of each. In addition, skin test assays could be carried out using these recombinant allergens to determine if they would be recognised *in vivo*. Furthermore, it may be useful to know when certain genes are “switched on” during mite development. This could be done by preparing mRNA from different stages of mites and conducting SAGE (serial analysis of gene expression) analysis. This method would allow the identification of those genes that

were up or down regulated in the various life stages of the parasite. However, to do this experiment one would require a readily available supply of mites from each different instar of development, which is not feasible at present as *P. ovis* mites cannot, as yet, be maintained in a laboratory environment.

CHAPTER 6: SEQUENCE ANALYSIS OF CYSTEINE PROTEASES

6.1 INTRODUCTION

Cysteine proteases have been identified from animals, plants and viruses. There are many types of cysteine proteases and they have been grouped into 27 clans, with the clans being further divided into families (Rawlings & Barrett, 1994). One of the major families is the papain superfamily, which includes many cysteine proteases of parasite origin. The biological activity of the cysteine proteases is dependent on a catalytic dyad of cysteine and histidine and a highly conserved asparagine (Rawlings & Barrett, 1994). There are two main types of proteases in the papain class: the cathepsin B proteases and the ERFNIN family, which includes the cathepsin L proteases. The cathepsin B family is characterised by both endopeptidase and exopeptidase dipeptidyl peptidase activity and the presence of an occluding loop (Karrer, Peiffer, & DiTomas, 1993). The occluding loop is an extra structural element not found in other papain-like proteases that blocks off one end of the substrate binding cleft and thus contributes to substrate specificity (Illy, Quraishi, Wang et al., 1997). The ERFNIN family are characterised by an interspersed conserved region which is formed over 20 bases and generally, has the structure: Glu, X₃, Arg, X₂, Ile/Val, Phe, X₂, Asn, X₃, Ile, X₃, Asn, where X denotes any amino acid (Karrer, Peiffer, & DiTomas, 1993).

Cysteine proteases have been widely studied in many parasite species and one of the main reasons is the belief that they may make good targets for chemotherapeutic attack. Cysteine proteases are considered essential for many functions associated with parasite survival e.g. digestion (Wasilewski et al., 1996), development (Richer, Hunt, Sakanari et al., 1993; Hashmi, Britton, Liu et al., 2002) and immunomodulation (Prowse, Chaplin, Robinson et al., 2002). Furthermore, the major house dust mite allergen, the group 1 allergens, are cysteine proteases (Chua et al., 1988), and this protease activity is thought to contribute to their allergenicity (Shakib & Gough, 2000).

Cysteine proteases have been shown to be effective vaccine candidates. For example, vaccination of cattle with cysteine proteases from the trematode *Fasciola hepatica* resulted in significant levels (50% to 73% reductions in fluke burdens) of protection (Wijffels et al., 1994; Dalton et al., 1996a; Robertson, Coombs, North et al., 1996). Vaccination of sheep with a cysteine protease-enriched fraction from *H. contortus* also stimulated useful levels of protective immunity (Knox, Smith, & Smith, 1999). Recent work with cysteine protease inhibitors has shown that they can block haemoglobin degradation in schistosomes *in vitro* and immunisation of mice with cysteine protease inhibitors resulted in a significant reduction in worm burden and egg production (Wasilewski et al., 1996).

The disease caused by infestation with the sheep scab mite, *Psoroptes ovis*, is thought to be an allergic type 1 hypersensitivity reaction, in response to products which are secreted or excreted by the mites. Earlier work in this project has identified four cysteine proteases present in the sheep scab mites (Chapter 5) cathepsin B1 and B2, cathepsin L and a homologue of the house dust mite group 1 allergen. The aim of this study was to obtain full-length sequence data for each of the cysteine proteases, then analyse the sequences to identify domains of interest and conserved residues that might provide an indication of their function, and confirm the class of cysteine protease identified. The clone encoding cathepsin B1 could not be recovered from the available glycerol stocks and was excluded from this analysis.

6.2 RESULTS

Full-length sequence for each of the cysteine proteases identified in the EST analysis (cathepsin B2, cathepsin L and a homologue of the house dust mite group 1 allergen) was derived using PCR and gene-specific sense or antisense primers, as described in section 2.7.1. Protein database searches (Blast, section 2.7.2) were carried out, then conserved motifs or domains of interest were identified using either the SMART or PIR Pattern Search websites, as described in section 2.7.2.

6.2.1 Sequence Analysis of Pso CathL1

The full-length sequence of the cathepsin L-like cysteine protease, Pso CathL1 is 1.016 kb and, when translated, predicts a protein of 338 amino acids. The translated sequence is shown in Figure 6.1. There is a signal peptide present from amino acids 1 – 21 (highlighted in dark blue in Figure 6.1). The sequence contains several features characteristic of cysteine proteases, including three active site domains, cysteine (pink), histidine (green) and asparagine (turquoise), where the actual active site residues are shown in bold (Figure 6.1). An ERFNIN domain is also present that is characteristic of non-cathepsin B cysteine proteases (Karrer, Peiffer, & DiTomas, 1993). The protein sequence was identified as a cathepsin L cysteine protease by database searches and the sequence was aligned with other cathepsin L proteins (Figure 6.2). The *P. ovis* cathepsin L sequence shares high levels of homology to other cathepsin L sequences, for example, 58% identity and 75% similarity with the cathepsin L cysteine protease from *Artemia sanfranciscana*, the brine shrimp. Uniquely, the *P. ovis* cathepsin L sequence contains a putative zinc-binding domain at residues 200 to 210.

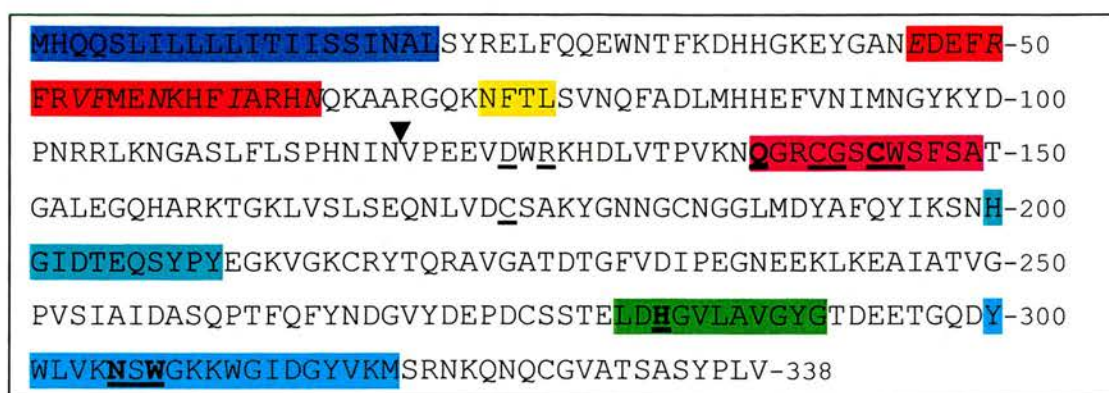


Figure 6.1. The protein sequence of Pso CathL1, a cathepsin L-like cysteine protease showing highlighted domains of interest and active site residues. Key: ■ -signal peptide; ■ - "ERFNI" domain – conserved amino acids in italics; ■ -N-glycosylation site; ■ -cysteine, ■ -histidine and ■ -asparagine active site domains conserved in cysteine proteases; ■ - zinc carboxypeptidase, zinc-binding region 2 signature; ▼ - putative protease cleavage site to yield mature enzyme; underlined residues - conserved amino acids in all cysteine proteases (Karrer, Peiffer, & DiTomas, 1993); residues in bold - active site amino acids (Karrer, Peiffer, & DiTomas, 1993); numbers at the end of each line indicate the number of amino acids.

20 ↓ 40

S.per: --MRT-VLVALALVALTQAISPLDLIKEEWHTYKLRKKNYANEVEERERMKLE : 52
Droso: --MRTAVLLPLALLAVAQAVSFADVMEEWHTFKLEHRKNYQDETEERERLKLE : 53
S.zea: ----MKLFLILAAVVISQAVSFYDLVQEQWSSEKMQHKNYDSETEERERMKLE : 51
A.san: MKQITLIFLLGAVLVQLSAALSLTNLLADEWHLEKATHKKEYPSQLEEKERMKLY : 55
B.mic: ----MLRLSVLCAIVAVTVAAASSQEILRTQWEAEKTTTHKKSQSHMEELLRFKLE : 51
PsoCL: MHQQSLILLLLITIISINALSYRELFFQEWNTFKDHHGKEYGANED-ERFRV : 54

60 80 100

S.per: NENRHKIAKHNLFAQGVSYKGLNKNYADMLHHEFKETMNGYNHTLRQLMRERT : 107
Droso: NENKHKIAKHNRFAEGKVSFKLAVNKNYADMLHHEFRQLMNGFNNTLHKQLRAAD : 108
S.zea: MENAKHVAKHNLFSQGFVKFKGLNKNYADMLHHEFVSTLNGFNKTKNNILKGS : 106
A.san: LENKHKVAKHNLILEYKGEKSYQVAMNKFGLHHEFRSIMNGYQHKKQNSSRAES : 110
B.mic: TENSIIIAKHNAKYAKGLVSYKGLMNQFGDLLAHEFARIFNGHHGTRKTG----- : 101
PsoCL: MENKHFIARHNQKAARGQKNFTLSVNQFADLMHHEFVNIMNGYKYDPNRRLLKNGA : 109

120 ↓ 140 160

S.per: G-LVGATYIPPAHVT---VPKSVDRWEHGAVTGVKDQGHCGSCWAFSSSTGALEGO : 158
Droso: ESFGKVTIIPPAHVT---LPKSVDRWTKGAVTAVKDQGHCGSCWAFSSSTGALEGO : 160
S.zea: L-NDAVREIIPANVK---LPDVTVDWRDKGAVTEVKDQGHCGSCWSFSATGSLEGO : 157
A.san: T----FTMEPANVE---VPESVDWRVKGAITPVKDQGCQSCWAFSSSTGALEGO : 158
B.mic: ----GSTELPPANVNDSSLPKVVDWRKKGAVTPVKDQGCQSCWAFSATGSLEGO : 152
PsoCL: S-----LFLSPHNIN---VPEEVDWRKHDLVTPVKNGRCGSCWSFSATGALEGO : 156

180 200 220

S.per: HFRKAGVLVSLSEQNLVDCSTKYGNNGCNGGLMDNAFRIYKDNKGIDTEKSYPYE : 213
Droso: HFRKSGVLVSLSEQNLVDCSTKYGNNGCNGGLMDNAFRIYKDNKGIDTEKSYPYE : 215
S.zea: HFRKTGKLVSLSEQNLVDCSGRYGNNGCNGGLMDNAFRIYKDNKGIDTEKSYPYL : 212
A.san: TFRKTGKLISLSEQNLIDCSGKYGNEGNGGLMDQAFQYIKDNKGIDTENTYPYE : 213
B.mic: FELKNGELVSLSEQNLVDCSQSFGNGGCEGGLMEDAFKYIKANDGIDTEKSYPYE : 207
PsoCL: HFRKTGKLVSLSEQNLVDCSAKYGNNGCNGGLMDYAFQYIKSNHGIDTEQSYPYE : 211

240 260

S.per: GIDDSCHFKNKATIGATDTGFVDIPEGDEEKMKKAVATMGPVSVVAIDASHESFQLY : 268
Droso: AIDDSCHFNGKGTVGATDRGFTDIPQGEKKMAEAVATVGPVSVVAIDASHESFQFY : 270
S.zea: AEDEKCHYKAQNSGATDKGFTDIEEANEDDLKAAVATVGPVSVVAIDASHETFQLY : 267
A.san: AEDNVCRYNPRNRGAIDRGFVHIPSCEEDKLKAAVATVGPVSVVAIDASHESFQFY : 268
B.mic: AVDGEGRFKKEDVGATDTGYVEIKAGSEVDLKKAVATVGPVSVVAIDASHSSFQLY : 262
PsoCL: GKVVGKCRYTQRAVGATDTGFVDIPEGNEEKLKEAIAVATVGPVSVVAIDASQPTFQFY : 266

280 300 320

S.per: SEGVYNEPECDEQNLDHGVLVVGFGTD-ESGMDYWLKNSWGTTWGEQGYIKMAR : 322
Droso: SEGVYNEPQCDAQNLDHGVLVVGFGTD-ESGEDYWLKNSWGTTWGDKGFIKMLR : 324
S.zea: SDGVYSDPECSSQELDHGVLVVGFGTS-DDGQDYWLKNSWGSPSWGLNGYIKMAR : 321
A.san: SKGVYVEPSCDSDLDHGVLVVGFGS--DNGQDYWLKNSWSEHWGDEGYIKIAR : 321
B.mic: SEGVYDEPECSSDLDHGVLVVGFGV--KGKKYWLKNSWAEWGDQGYILMSR : 315
PsoCL: NDGVYDEPDCSSTELDHGVLVVGFGTDEETGQDYWLKNSWGKKWGIDGYIKMSR : 321

340

S.per:	NQNNQCGIATASSYPTV	: 339
Droso:	NKENQCGIASASSYPLV	: 341
S.zea:	NQDNMCGVASQASYPLV	: 338
A.san:	NRKNHCGIATAASYPLV	: 338
B.mic:	DNNNQCGIASQASYPLV	: 332
PsoCL:	NKQNNQCGVATSASYPLV	: 338

Figure 6.2 Alignment of the cathepsin L-like cysteine proteases. Residues in black are 100% identical those in grey are 80% similar. -, gaps inserted into sequence to improve alignment. *, denotes active site residues. The sequences used and their GenBank sequences are: PsoCL, Cathepsin L1 from EST analysis, BQ834906; A.san, *Artemia sanfranciscana* (brine shrimp), Q9Y0X2; S.per, *Sarcophaga peregrina* (flesh fly) Q26636; Droso, *Drosophila melanogaster*, Q95029; S.zea, *Sitophilus zeamais*, O46030; B.mic, *Boophilus microplus*, AF227957. ↓ indicates predicted signal peptide and mature protease cleavage sites (Renard, Garcia, Cardoso et al., 2000).

6.2.2 Sequence Analysis of Pso CathB2

The full-length sequence of the cathepsin B-like cysteine protease, Pso CathB2 is 1.019 kb in length, which, when translated results in a predicted protein sequence of 339 amino acids. The translated sequence is shown in Figure 6.3 with domains of interest and conserved residues highlighted. Again, the protein can be confirmed as a cysteine protease due to the presence of cysteine, histidine and asparagine active sites residues. A signal peptide is also predicted at amino acids 1-18, and there is a putative N-glycosylation site present at amino acids 66-70 (Figure 6.3). The cathepsin Bs are characterised by the presence of an occluding loop. Pso CathB2 incorporates the signature sequence for an occluding loop from amino acids 188-214 (highlighted in red, Figure 6.3). The alignment of the protein sequence of Pso CathB2 with other cathepsin B proteases (Figure 6.4) shows limited homology in the prepro- region but this increases markedly in the sequence encoding the mature enzyme protein.

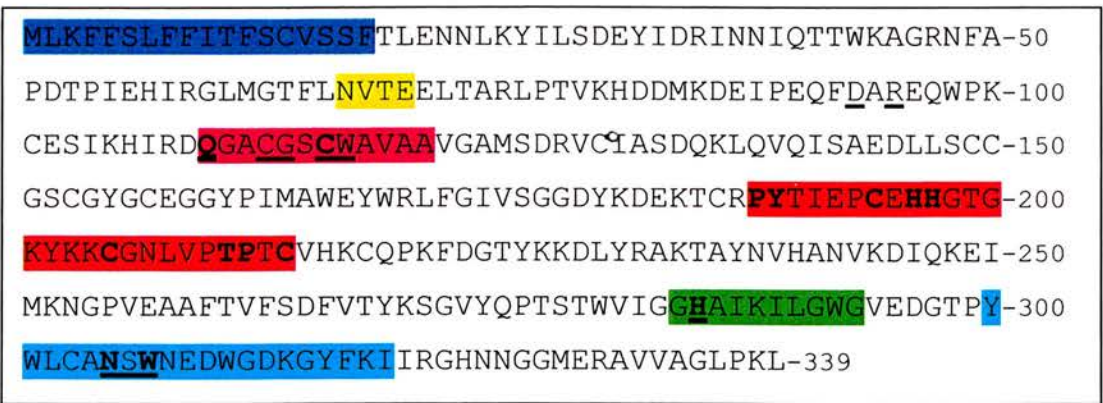


Figure 6.3 Deduced protein sequence for Pso CathB2, the cathepsin B-like cysteine protease. Key: ■-Signal peptide; ■-cysteine, ■-histidine and ■-asparagine active site domains conserved in cysteine proteases; ■-N-glycosylation site; ■-occluding loop sequence; underlined residues -conserved amino acids in all cysteine proteases (Karrer, Peiffer, & DiTomas, 1993); residues in bold - active site amino acids (Karrer, Peiffer, & DiTomas, 1993); numbers at the end of each line denote the number of amino acids.

20 40

Bomby: ----MFISRAAYVTLVCVLA--AAKDLPPPLSDEFINTINLKQNS-WKAGRNFPR : 48
Helic: ----MAASRATFVALVCALAL-AAADVQNPLSDDFINLINTKQNS-WKAGRNFPE : 49
Human: ----MWQLWASLCCLLVLANA-RSRPSFHPVSDELVNYVVKRNTT-WQAGHNFYN : 49
PovCB: ---MLKFFSLFFITFSCVSSFTLENNLKYYLSDEYIDRINNIQTT-WKAGRNFAP : 51
Sarco: MRQHFVVIICIAFLAFGQVLAN-LDAEN-DLISDEFLEIVRSKAKT-WTPGRNYDK : 52
Schja: -MLKIAVYIVSLFTTFLEAHVTTNRNQRIEPLSDEMISFINEHPDAGWKADK-SDR : 53
Schma: -MLTSILCIASLITFLEAHISVKN-EKFEPLSDDIISYINEHPNAGWRAEK-SNR : 52

60 80 100

Bomby: DTSFAHLKKIMGVIEDEHFATLPIK---THKIDLIAG-LPENFDPRDKWPCPT : 98
Helic: HTPFAHIKRLAGVLPDYHLSKLSKV---EHEDELIAS-LPENFDPRDKWPCPT : 99
Human: -VDMSYLKRLCGTFLGGPKPPQVRMF---TEDLKLPA-----FDAREQWPQCPT : 95
PovCB: DTPIEHIRGLMGTFLNVTEELTARLP---TVKHDDMKDEIPEQFDAREQWPKCES : 103
Sarco: SVPRSHFRRLMGVHPDAHKTFLHEKSLVLGEEVGLADSDVPEEFDARKAWPNCPT : 107
Schja: FHSLDDARILMGARKEDAEMKRNRRP---TVDHDLNVEIPSQFDSRKKWPHCKS : 105
Schma: FHSLDDARIQMGARREEDLRRKRRP---TVDHNDWNVEIPSNFDSRKKWPGCKS : 104

120 140 160

Bomby: LNEVRDQSGCGSCWAFGAVEAMTDRVCTYSNGTKHFHFAEDLLSCC-PICGLGC : 152
Helic: LNEVRDQSGCGSCWAFGAVEAMTDRYCTYSNGTQHFHFAEDLLSCC-PICGLGC : 153
Human: IKEIRDQSGCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTCCGSMCGDGC : 150
PovCB: IKHIRDQGACGSCWAVAAVGAMSDRVCIASDQKLQVQISAEDLLSCC-GSCGYGC : 157
Sarco: IGEIRDQSGCGSCWAFGAVEAMSDRLCIHSNATIHFHFSADDLVSCC-HTCGFGC : 161
Schja: ISQIRDQSRGCGSCWAFGAVEAMTDRICTISGGGQSAELSALDLISCC-KDCGDGC : 159
Schma: IATIRDQSRGCGSCWAFGAVEAMSDRSCTISGGKQNVELSAVDLLTCC-ESCGLGC : 158

180 200 220

Bomby: SGGMPRLAWEYWKHFGLVSGGSYNSSQGCRPYEIPPCEHHVPGNRMPCSGDT-KT : 206
Helic: NGGMPRLAWEYWKHFGLVSGGSYNSSQGCRPYEIPPCEHHVPGNRMPCNGDS-KT : 207
Human: NGGYPAEAWNFWRKGLVSGGLYESHVGCRRPYSIIPPCEHHVNGSRPPCTGEG-DT : 204
PovCB: EGGYPIMAWEYWRFLGIVSGGDYKDEKTCRPYTIIEPCEHHGTGKYKKC-GNLVPT : 211
Sarco: NGGFPGAAWAYWTRKGLVSGGPGSSQGCRPYEIIAPCEHHVNGTRPPCDGEHGKT : 216
Schja: QGGFEGVANDYWKRGIVTGGSKENHTGCQPYPPFKCEHHTKGKYPACGTKIYKT : 214
Schma: EGGILGPANDYWKREGIVTASSKENHTGCEPYPPFKCEHHTKGKYPGSKIYNT : 213

240 260

Bomby: PKCTKKCESGIDVNYKQDKQYCKHVTVSGDEDHIRAE LFKNGPVEGAFTVYSDL : 261
Helic: PKCEKTCESNINVDYRKDKRYGKHVFSVSSKEDHIRAE LFKNGPVEGAFTVYSDL : 262
Human: PKCSKICEPGISPTYKQDKHYGYSYSVSNSEKDIMA EYKNGPVEGAFTVYSDF : 259
PovCB: PTCVHKCQPKFDGTYKKDLYRAKTAINVHANVKDIQKEIMKNGPVEAAFTVFSDF : 266
Sarco: PSCRHECQKSYDVDYKTDKHFCSKSYSVKRNVKDIQKEIMQNGPVEGAFTVYEDL : 271
Schja: PQCKQTCQKGYKTPYEQDKHYGDESINVQNEKVIQORDIMMYGPVEAAFDVYEDF : 269
Schma: PRCKQTCQKGYKTPYTQDKHRGKSSYNVKNDEKAIQKEIMKYGPVEASFIVYEDF : 268

280 300 320

Bomby: LSYKSGVYKHTQCDALGGHAVKILGWGVENDNKYWL IANSWN SDWGDNGFFKILR : 316
Helic: LNYKTGVYKHTICDALGGHAVKILGWGVENGKNKYWL IANSWN SDWGDNGFFKILR : 317
Human: LLYKSGVYQHVTGEMMGHAIIRILGWGVENGTPYWL VANSWNTDWDGNGFFKILR : 314
PovCB: VTYKSGVYQPTSTWVIGGHAIRILGWGVEDGTPYWL CANSWNEDWDGDKYFKIIR : 321
Sarco: ILYKDGVIYQHVHGRELGHAIRILGWGVENKTPYWL IANSWNTDWDGNNGFFKMLR : 326
Schja: LNYKSGIYRHVTSIVGGHAIRIIGWGVENKTPYWL IANSWNEDWGEKGLFRMVR : 324
Schma: LNYKSGIYKHITGEALGGHAIRIIGWGVENKTPYWL IANSWNEDWGENCYFRIVR : 323

340

```

Bomby: GEDHCGTIESSIVTGEPLDEH---- : 337
Helic: GEDHCGTIESSIVAGEPMFVEY---- : 338
Human: GQDHCGTIESEVVAGIPRTDQYWEKI : 339
PovCB: GHNNGGMERAVVAGLPKL----- : 339
Sarco: GEDHCGTIESAIAAGLPKV----- : 344
Schja: GRDECSLESVDVAGLIKT----- : 342
Schma: GRDECSLESEVIAGRIN----- : 340

```

Figure 6.4 The alignment of the cathepsin B cysteine proteases. Residues in black are 100% identical those in grey are 80% similar. -, indicates gaps inserted to improve the alignment. *, active site amino acids. ^, denotes the conserved residues of the occluding loop; ↓ indicates the likely cleavage site to yield the mature protease. The sequences used and their GenBank accession numbers are as follows: Bomby, *Bombyx mori*, Q9BLI9; Helic, *Helicoverpa armigera* (cotton bollworm), Q9NHF5; Sarco, *Sarcophaga peregrina* (flesh fly), Q26655; Human, P07858; PovCB, *Psoroptes ovis* Pso CathB2, BQ834913; Schja, *Schistosoma japonicum*, P43157; Schma, *S. mansoni*, P25792.

6.2.3 Sequence Analysis of Pso Der f A

The full length *P. ovis* sequence for the *Der f 1* homologue (Pso Der f A) is 969 bp in length and encodes a predicted protein of 322 amino acids. The protein sequence of Pso Der f A is shown in Figure 6.5, with domains of interest and active sites highlighted. A predicted signal peptide is present from amino acids 1-19 (Figure 6.5, dark blue highlighted area). The sequence contains the characteristic cysteine protease active site residues, namely cysteine (pink), histidine (green) and asparagine (turquoise). The active site residues are shown in bold (Figure 6.5). An N-glycosylation site is present from amino acids 152-155. There does not appear to be an ERFNIN domain or occluding loop signature site present. A putative IgE binding site is present from amino acids 251 to 257 (red highlighted area, Figure 6.5). An alignment of the protein sequence of Pso Der f A with the protein sequences of the other group 1 allergens is shown in Figure 6.6. The Pso Der f A sequence is highly homologous to the *Der f 1* sequence, sharing 67% identity and 78% similarity.

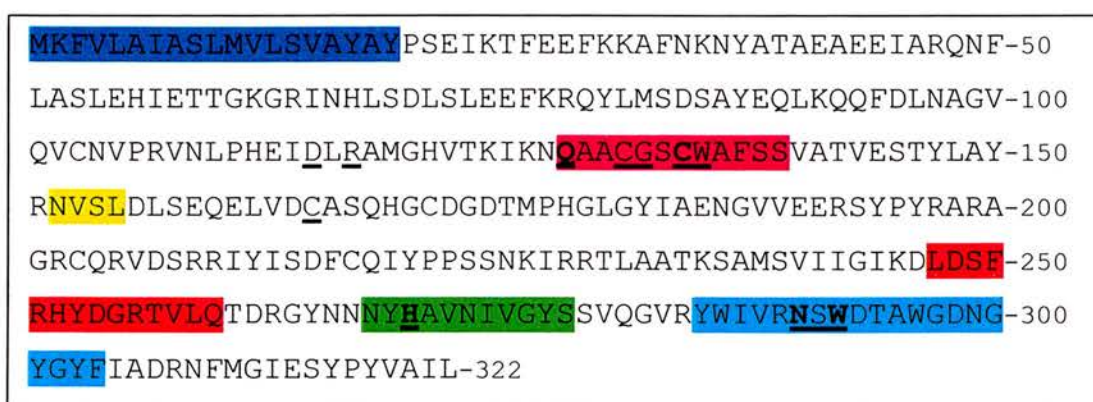


Figure 6.5 Protein sequence of Pso Der f A, the *Der f 1* homologue, showing domains and active sites of interest. Key: ■-Signal peptide; ■-cysteine, ■-histidine and ■-asparagine active site domains conserved in cysteine proteases; ■-N-glycosylation site; ■- putative IgE binding site; underlined residues -conserved amino acids in all cysteine proteases (Karrer, Peiffer, & DiTomas, 1993); residues in bold - active site amino acids (Karrer, Peiffer, & DiTomas, 1993); numbers at the end of each line indicate the number of amino acids.

6.2.4 Phylogenetic Analysis of *P. ovis* Cysteine Proteases

Although the group 1 allergens have been defined as cysteine proteases (Chua et al., 1988), the class of cysteine protease to which they belong remains unclear. The Pso Der f A sequence was aligned with both a cathepsin B and a cathepsin L sequence. This analysis indicated that Pso Der f A had 25% identity and 40% similarity to a human cathepsin L sequence (Figure 6.7) and 17% identity and 32% similarity with a cathepsin B sequence from *Ascaris suum* (Figure 6.8). There did not appear to be An ERFNIN domain, which is indicative of most non-cathepsin B proteases, was absent but the signature of an occluding loop, which is characteristic of cathepsin Bs, was also lacking. This suggested that the Pso Der f A sequence was neither a cathepsin B-like protease nor a member of the ERFNIN family of cysteine proteases which include cathepsin L. Therefore, in order to further define what type of cysteine proteases the group 1 allergens are, phylogenetic analysis was carried out (Figure 6.9). Each of the families of cysteine protease form distinct clades, indicating that they are more closely related to each other than they are to members of different families. Each of the families of cysteine proteases has been highlighted in the phylogram and the *P. ovis* sequences have been boxed to identify them from the others. The *P. ovis* cathepsin L-like protease is found in the clade with other members of the cathepsin L cysteine proteases, and the *P. ovis* cathepsin B-like clone is grouped with other cathepsin Bs. It is interesting to note that the group 1 allergens are grouped together, separate from the other proteases. This grouping is highlighted in yellow in Figure 6.9.

```

                20                      40
CathL-hum: MNPTLILAAFCGLGIASATLTFDHSLEAQWTKWKAMHNRLYGMNEEGWRRRA : 50
Pov_Derfa: --MKFVLAIASLMVLSVAYAYPSEIKTFEEFKKAFNKNYATAEAEIARQ : 48

                60                      80                      100
CathL-hum: VWEKNMKMTIELHNQYREGKHSFTMAMNAFGDMTSEEFQVMNGFQNRKP : 100
Pov_Derfa: NFLASLEHLETGTGGRINHLSDLSLEEFKRQYLMSSDAYEQLK--QQFDL : 96

                120                      140
CathL-hum: RKCKVFQEPLFYEA PRSVDWREKGYVTPVKNQGC GSCWAFSATGALEGQ : 150
Pov_Derfa: NAGVQVCNVP RVNLPHEIDL RAMGHVT KIKNQAACGSCWAFSSVATVEST : 146
                * *

                160                      180                      200
CathL-hum: MFRKTGR LISLSEQNLVDCSGPQGNEGCNGGLMDYAFQYVQDNGGLDSEE : 200
Pov_Derfa: YLAYRNVSLDLSEQELVDCASQHG---CDGDTMPHGLGYIAENG VVE-ER : 192

                220                      240
CathL-hum: SYPYEATEESCK-YNPKYSVANDTGFVDIPKQEKALMKAVATVGPISVAI : 249
Pov_Derfa: SYPYRARAGRCQRVDSRR IYISDFCQIYPPSSNKIRRTLAATKSAMSVII : 242

                260                      280                      300
CathL-hum: DAG-HESFLFYKEGIYFEPDCSSEDMDHGVLVVG YGFESTESDNNKYWL V : 298
Pov_Derfa: GIKDLDSFRHYDGRTVLQTD RGYNNNYH AVNIVGY----SSVQGVRYWIV : 288
                *

                320
CathL-hum: KNSWGEETWGMGGYVKMAKDRRNHCGIASAASYPTV : 333
Pov_Derfa: RNSWDTAWGDNGYGYFIADR-NFMGIESYPYVAIL : 322
                * *

```

Figure 6.7 Alignment of the *P. ovis* Der f A (accession number BQ834852) and the human cathepsin L cysteine protease (accession number P07711). Residues shaded in black are 100% identical. -, gaps added to improve sequence alignment. *, indicates active site residues. The ERFNIN domain residues are highlighted in grey on the human cathepsin L sequence.

```

                20                      40
CathB-asca: MRYTFLVALLAIAANSSADRRHLTDDSSSSDESYGSRYYGDGRSVLDEFRD : 50
Povis_Derf: MKFVLAIASIMVLSVAYAYPSEIKTFEEFKKAFNKNYAT--AEAEETIAR : 47

                60                      80                      100
CathB-asca: RKISHEAEKLTGYALANYVNRKQNLWKAKFNNKFRNYSDRVKYGLMGVNN : 100
Povis_Derf: QNFLASLEHIETTGKG-RINHLSDLSEEFKQRYLMSDS-----AYEQ : 89

                120                      140
CathB-asca: VRLSVKAKKNLSPTRFYDIYIPEAFDAREKWDQCASLKNIRDOSSCGSCW : 150
Povis_Derf: LKQQFDLNAGVQVCNVP RVNLPHEIDL RAMG---HVTIKIKNOAACGSCW : 135
                                         *      *

                160                      180                      200
CathB-asca: AFGAVEAMSDRICIASNGKIQVSLSADDLLSCCKSCGFGCDGGDPMAAWK : 200
Povis_Derf: AFSSVATVESTYLAYRN--VSLDLSEQELVDCAS--QHGCDDGTMPHGLG : 181

                220                      240
CathB-asca: YWVKEGIVTGSNFTMKQGCKPYFPFPCHEHHSNKTHYQPCKHDLYPTPKCE : 250
Povis_Derf: YIAENGVVEERSYPYRARG-----RCQRVDSRRIYISDFCQIYPP---- : 222

                260                      280                      300
CathB-asca: KKCLDIYTEKTYAEDKFFGETAYGVEDDVTSIQKEILTHGPIVEVAFEVYE : 300
Povis_Derf: -----SSNKIRRTLAATKSAMSVIIGIKDLD : 248

                320                      340
CathB-asca: DFLMYDGGIYVHTG-GKIGGGHAVKMLGWGVEQGVPYWLIVANSWNTDWGE : 349
Povis_Derf: SFRHYDGRTVLQTDRCYNNNYHAVNIVGYSSVQGVRYWIVRNSWDTAWGD : 298
                        *                                * *

                360                      380
CathB-asca: DGFFRIIRGIDECGIESVVGGLPKLNRTYKKYHRRYRLDNDEDDDIIF : 398
Povis_Derf: NGYGYFIADRNFMGIESYPYVAIL----- : 322

```

Figure 6.8 Alignment of Pso CathB2 and cathepsin B from *Ascaris suum* (accession number U51892). Residues shaded in black are 100% identical. -, gaps inserted to improve the alignment. *, denotes active site residues. The occluding loop conserved residues are highlighted in grey in the *A. suum* sequence.

6.3 DISCUSSION

The aim of this study was to obtain full-length sequence data for each of the cysteine proteases identified from the EST analysis. Detailed sequence analysis was then carried out to identify conserved domains that may give an indication of the function of these proteases. The study also aimed to confirm the class of cysteine proteases to which the *P. ovis* proteases belong. The work was carried out on three cysteine protease sequences, namely Pso Der f A, the group 1 mite allergen homologue, Pso CathB2 and Pso Cath L1 (Table 5.1, Chapter 5). Pso CathB1 was not studied as the clone could not be recovered from the glycerol stocks (Chapter 2, section 2.6.2).

Full-length nucleotide sequence for each of the three proteases was identified using PCR analysis. The amino acid sequence was then deduced from the nucleotide sequence and used for the comparisons and domain searches. Each of the sequences could be determined as cysteine proteases due to the presence of three conserved active site residues, cysteine, histidine and asparagine (Figures 6.1, 6.3 and 6.5). These active site residues are present in all cysteine proteases and form the catalytic triad (Karrer, Peiffer, & DiTomas, 1993).

The Pso CathL1 sequence was defined as a cathepsin L-like protease by database searches and the presence of an ERFNIN domain (Figure 6.1). The presence of a signal peptide suggests that this protease may be secreted by the mite. The Pso CathL1 sequence contains a putative zinc-binding domain **HGidtEqsYPY** (residues 200 – 210), which is a feature not previously described in the cathepsin Ls. In the alignment shown (Figure 6.2), the motif is conserved amongst all the sequences tested with the notable exception of the histidine residue, which is unique to the *P. ovis* sequence. Histidine residues can donate electrons while their side chains are able to adopt favourable conformations to form metal binding sites (Willett, Gillmor, Perona et al., 1995). It is unclear whether Pso CathL1 shows metal ion dependent protease activity, but this could be tested by measuring protease activity in mite extracts using cathepsin L-specific peptide substrates in the presence or absence of chelating agents.

Cathepsin L proteases have recently been identified from the cattle tick, *Boophilus microplus* (Renard et al., 2000), but most of the work in multicellular parasites to date has concentrated on the helminth parasites, *Fasciola hepatica*, *Haemonchus contortus* and *Schistosoma* spp. For example, cathepsin L proteases have been identified from *H. contortus* (Rhoads & Fetterer, 1995), *F. hepatica*, FheCL1 (Smith et al., 1993b) and FheCL2 (Dowd et al., 1994), and from *Schistosoma mansoni*, SmCL1 and SmCL2 (Dalton, Clough, Jones et al., 1996b). These proteases are thought to be involved in digestion, but also may have other roles. For example, *H. contortus* cathepsin L is expressed by cells lining the intestine, and is thought to have a role in digestion of host haemoglobin (Rhoads & Fetterer, 1995). More recently, a cathepsin L that is essential for embryogenesis was identified in *C. elegans* and transgenic studies showed that other parasitic nematodes, including *H. contortus*, contained functional orthologues (Britton & Murray, 2002). Therefore, cathepsin Ls may serve a dual function in nutrition and reproduction. This dual role is also suggested in the trematode parasite *S. mansoni* where two cathepsin L encoding genes have been identified. The first, SmCL1 (Brady, Dowd, Brindley et al., 1999) may facilitate haemoglobin degradation while the second, SmCL2, localises to the reproductive organs of *S. mansoni* males and females, with more transcripts being found in females (Michel, Ghoneim, Resto et al., 1995). The authors suggest that these proteases are secreted and may have a role in the passage of various reproductive cells through the ducts of the uterus. FheCL1 and FheCL2 are thought to be involved in digestion (Howell, 1966; Halton, 1967). FheCL1 has also been implicated in the cleavage of host IgG (Smith et al., 1993a) and the prevention of antibody-mediated attachment of host eosinophils to newly excysted juveniles (Carmona, Dowd, Smith et al., 1993). FheCL2 was found to cleave host fibrinogen, which results in the production of a novel fibrin clot (Dowd, McGonigle, & Dalton, 1995). These cathepsin L proteases also have immunomodulatory effects that include the reduction of sheep T-cell proliferation and the down regulation of surface CD4 expression in both human and ovine cells (Prowse et al., 2002). It is thought that these immunomodulatory roles aid in parasite development in the host.

Cathepsin L enzymes have been shown to be effective vaccine candidates against fasciolosis in sheep (Wijffels et al., 1994; Piacenza et al., 1999), and cattle

(Dalton et al., 1996a; Mulcahy, O'Connor, Clery et al., 1999). Significant protection was achieved with immunisation with cathepsin L and this was found to be enhanced if multiple antigens were used simultaneously (Dalton et al., 1996a). The vaccination of animals with these proteases resulted in a significant reduction in the number of viable eggs produced per adult (Wijffels et al., 1994).

Cathepsin B cysteine proteases are unique within the papain class due to their endopeptidase and peptidyl dipeptidase activity (Aronson & Barrett, 1978; Bond & Barrett, 1980; Karrer, Peiffer, & DiTomas, 1993; Rawlings & Barrett, 1994). The peptidyl dipeptidase activity results in proteolytic release of dipeptides from the C-terminus. This activity is thought to occur due to the presence of an extra insertion termed the occluding loop which is composed of approx. 20 residues and is characterised by double histidine motif (Musil, Zucic, Turk et al., 1991; Rehman & Jasmer, 1999). The occluding loop is thought to be important in substrate specificity, as its position obscures one end of the substrate binding cleft from macromolecules. The *P. ovis* cathepsin B has the sequence signature for an occluding loop at amino acids 188 to 214 (Figure 6.5) and several residues are conserved when compared to other cathepsin B-like cysteine proteases (Figure 6.6).

Further studies are required to determine if the Pso CathB2 sequence has dipeptidyl peptidase activity. To do this the recombinant protein would need to be expressed in a suitable vector, e.g. a bacterial plasmid such as pET or the commonly used yeast expression system *Pichia pastoris*, and enzyme activity determined using a suitable specific assay.

A predicted signal peptide is also present from amino acids 1-18 (Figure 6.5), of Pso CathB2, which suggests that this protease may be secreted. In other parasites, cathepsin B proteases have been linked with immune evasion e.g. the 29 kDa protease from newly excysted juvenile *Fasciola hepatica* degrades sheep IgG (Wilson, Good, Panaccio et al., 1998). Cysteine proteases are thought to be linked with allergic responses against the hookworm, *Ancylostoma caninum*. Recently cathepsin B like proteases have been identified from E/S products, indicating they are secreted and may elicit the hypersensitivity responses observed (Harrop, Sawangjaroen, Prociv et al., 1995).

Cathepsin B-like proteases are showing promise as vaccine candidates. A cysteine protease-enriched protein sub-set prepared from adult *H. contortus* integral membrane protein extracts conferred substantial protection (50% and 70% reductions in worms numbers and faecal egg output, respectively) in vaccinated lambs given a single challenge infection of the same parasite, when compared with unvaccinated controls (Knox, Smith, & Smith, 1999). Recent experiments and analyses have shown that the cysteine proteases are all cathepsin B homologues (D.P. Knox, personal communication). The levels of protection observed would be sufficient to control haemonchosis if the same effect could be obtained with recombinant versions of the proteases.

The *P. ovis* group 1 allergen homologue, Pso Der f A, shows strong homology with the other group 1 allergens that have been identified from house dust mites, namely *Der f 1* from *Dermatophagoides farinae*, *Eur m 1* from *Euroglyphus maynei* and *Der p 1* from *D. pteronyssinus* (Figure 6.6). These sequences have been defined as cysteine proteases (Chua et al., 1988), and this study has shown that all the characteristic conserved domains are present in Pso Der f A (Figure 6.5). The group 1 allergens are extremely important in the house dust mite infection. The group 1 allergens are one of the major allergens, recognised by approx. 90% of mite allergic people, and bind IgE at a high frequency (Chua et al., 1988; Stewart, 1995).

A highly conserved domain (**LdsFrHYDGRTvIQ**), thought to be a putative IgE binding site, has been identified by comparison of molecular models of the mite group 1 allergens (Furmonaviciene, Sewell, & Shakib, 2000; Sajid & McKerrow, 2002). This domain was also highly conserved in the Pso Der f A sequence (Figure 6.5, amino acids 247-260). This putative IgE binding site was identified using molecular models of the group 1 house dust mite allergens, so whether this domain is a functional IgE binding site in Pso Der f A remains to be determined.

In *D. pteronyssinus*, *Der p 1* is found in high concentrations in mite faeces and is present in the mite gut epithelium (Tovey, Chapman, & Platts-Mills, 1981; Tovey & Baldo, 1990; Thomas, Heap, & Carswell, 1991). The enzyme is produced and secreted into the alimentary canal (Thompson & Carswell, 1988) suggesting a function as a digestive enzyme. There is now a large body of evidence that suggests there is a link between the cysteine protease activity of *Der p 1* and its allergenicity

(reviewed by Pomés & Chapman, 2001). This is true of other cysteine proteases e.g. the allergenicity of papain is closely linked to its enzymatic activity (Finkelman & Urban Jr, 1992).

Der p 1 is thought to be able to enhance IgE synthesis. For example, *Der p 1* can cleave CD23, the low affinity receptor for human IgE (Schulz et al., 1995; Hewitt et al., 1995). When IgE is bound to CD23 it is thought to protect CD23 from proteolytic cleavage and form a negative feedback loop to reduce IgE synthesis from B-cells (Sherr, Macy, Kimata et al., 1980, Sutton & Gould, 1993). However, when membrane CD23 is proteolytically cleaved it results in the release of soluble CD23 fragments. Soluble CD23 is thought to be important in stimulating growth and differentiation of the precursors of plasma cells, T-cells and basophils (Sutton & Gould, 1993), and thus the increase in soluble CD23 may up-regulate IgE synthesis.

Der p 1 can also cleave CD25, the alpha-subunit of the IL-2 receptor. CD25 is important in the propagation of Th1 cells, therefore its cleavage by *Der p 1* may bias the immune response towards Th2 cells, thereby creating an allergic environment (Schulz, Sewell, & Shakib, 1998). In addition, *Der p 1* can also promote type 2 responses by modulating the balance between IL-4 and IFN- γ (Comoy, Pestel, Duez et al., 1998). Recently, *in vivo* studies have been carried out that confirm these findings. Mice immunised with enzymatically active *Der p 1* showed a significant enhancement of total IgE and *Der p 1*-specific IgE responses. This effect was not observed in animals immunised with *Der p 1* that had been inactivated by inhibition with E64 (Gough, Schulz, Sewell et al., 1999), suggesting that the allergenicity of *Der p 1* is closely linked with its proteolytic activity.

Der p 1 can increase the permeability of bronchial epithelium, by the disruption of tight junctions, thereby increasing the permeability of the epithelium to macromolecules e.g. serum albumin (Herbert, King, Ring et al., 1995; Wan, Winton, Soeller et al., 1999). In addition, *Der p 1* can also cleave α -1-antitrypsin, an antiprotease that is thought to be involved in innate immunity on mucosal surfaces (Schulz et al., 1995), thereby facilitating its own passage through the epithelial layer. The proteolytic activity of *Der p 1* may also stimulate the release of proinflammatory cytokines from human bronchial epithelium. The deposition of mite proteases on the mucosal surface may induce a localised inflammatory response. The release of

cytokines (IL-6, IL-8 and GM-CSF) from epithelium may alter immunogenicity due to the effects that these cytokines have on several other processes, e.g. B-cell differentiation, chemoattraction of neutrophils and eosinophils (King, Brennan, Thompson et al., 1998). If the Pso Der f A protease has similar biological activities, this may explain the histological changes that occur directly after *P. ovis* mites are applied to sheep skin, e.g. the inflammatory response and influx of eosinophils.

The phylogenetic analysis conducted in this study suggests that the group 1 allergens form a separate clade from other cysteine proteases. In this study, the phylogenetic analysis was used purely to give an indication of the class of proteases these allergens may be part of. The fact that they form their own unique clade, suggests that the group 1 allergens may have domains or signatures that are unique to them, and therefore may be important in their observed allergenicity. Further work will be required to determine if there are unique domains or sequences associated with these highly allergenic molecules.

At present, the allergenicity of these proteases is undefined as they were isolated by EST analysis. Future work is therefore required to define the allergenicity of each of the proteases. Firstly, each protease must be expressed in a suitable expression vector, and then the recombinant protein can be used in ELISA to identify any IgE binding, or in skin test assays to determine if an allergic response can be observed in a sensitised animal.

This study has shown that there are at least three separate classes of cysteine protease present in the *P. ovis* mites, cathepsin B-like and cathepsin L-like cysteine proteases, as well as a homologue of a mite group 1 allergen. The group 1 allergens are extremely important in the house dust mite allergic response observed in humans and it is suggested that they may be equally important in the sheep scab mite infestation. Phylogenetic analysis of the cysteine proteases identified from *P. ovis* has shown that the cathepsin B and cathepsin L group in the same clades as other cathepsin Bs and Ls respectively. In addition, the phylogenetic analysis has shown the group 1 allergens form a unique class of cysteine proteases, which may also suggest that they have motifs or domains that are unique and determine their allergenicity.

CHAPTER 7: GENERAL DISCUSSION

As stated earlier, sheep scab is a highly contagious ectoparasitic disease of sheep that is caused by infestation with the mite, *Psoroptes ovis*. While the disease has economic consequences, the welfare issue surrounding this disease is of major importance due to the clinical symptoms induced, which include intense pruritis and severe exudative dermatitis. The disease is presently controlled by chemical intervention which is considered unsustainable because of the toxic nature to the operator of some of the compounds used, consumer concern regarding chemical residues in the meat and fleece as well as environmental issues. Perhaps the major long-term concern is the appearance of drug resistance in the mite population. By analogy to the situation developing in nematode populations with anthelmintics, now is the time to develop non-chemical based control strategies during a period when the available compounds maintain their efficacy.

Theoretically, it should be possible to eradicate sheep scab from the UK, but after a 20-year government controlled campaign, sheep scab is still rife in the UK. In 1992, the government control programme ceased when sheep scab was deregulated from a notifiable disease and the prevalence of the disease has been increasing throughout the UK since then (Coles, 1998; M. Taylor; personal communication). The unsustainable nature of the chemical treatments used to date and the continued prevalence of the disease mean that new methods of control are required. Sheep do become immune to *P. ovis* infestation, an observation that suggests that the development of a vaccine to control the disease is a theoretical possibility. Indeed, recent experiments have shown that immunisation of sheep with fractionated mite extract can stimulate significant protective immunity (Smith et al., 2002). While this result is encouraging, vaccinated sheep still carried mites and the lesion was reduced in size, not eliminated (Smith et al., 2002). In this situation, the farmer would still need to use chemical intervention to prevent the physical manifestations of infection. Hence, it could be anticipated that a vaccine that is not 100% effective might have more application as a control measure to reduce mite numbers and subsequent disease spread, with chemicals still being required to remove the “on sheep” population. Another possibility would be to “desensitise” sheep to mite allergens

using controlled exposure. In either case, the antigens associated with *P. ovis* remain largely undefined. The overall objectives of this project were to identify proteins that may be associated with the pathogenesis of the disease and to identify putative vaccine candidates.

Several proteins were identified that may have a role in mite physiology. The biochemical analysis described in Chapter 3 identified several proteases. Cysteine proteases dominated but aspartyl and metallo-proteases were also present. These proteases were capable of degrading several substrates that would be found in the mites natural habitat, sheep skin, e.g. fibrinogen, IgG, haemoglobin and collagen-based substrates. These results suggest that the proteases may have a role in mite physiology including roles such as digestion, anticoagulation and immune evasion. Although these putative functions have been suggested for the proteases present in *P. ovis*, they cannot be confirmed until further analysis has been carried out.

The presence of cysteine proteases in the mites was confirmed by the EST analysis (Chapter 5), which identified 4 different cysteine proteases, two cathepsin Bs, a cathepsin L and the mite group 1 allergen homologue, which is a cysteine protease.

Further analyses of the proteases could include the purification of the native enzymes and definition of their biochemical properties and substrate specificities to determine if they are allergens or useful immunogens. In addition, studies to identify the location of these proteases in the mites may provide an insight to their function. These analyses are compromised by the difficulties associated with acquiring sufficient mite material as a source of enzymes. One solution is to express the enzymes and other proteins of similar interest, in pro- (bacteria) or eukaryotic (yeast, insect cells, mammalian cell lines) expression systems. There are technical problems associated with all these systems, probably of most importance, achieving the appropriate post-translational processing (Dalton, Brindley, Knox et al., 2002), which directly affects function and immunogenicity.

The inhibition of protease activity by antibody may be a host-protective method to limit the damage caused by active proteases. Although, in this study, no inhibition of the proteases by antibody was observed *in vitro*, this cannot be ruled out until further, more sensitive, analyses have been conducted, such as the use of low

molecular weight synthetic peptide substrates as an alternative to protease gels. Another possibility is that acute phase proteins, such as alpha-2-macroglobulin, may be produced as part of the inflammatory response to the mite and effectively inhibits mite proteases. This possibility could be tested *in vitro* by incubating mite extracts with lesion washings prior to determining residual protease activity. Again, the protease assays could be carried out using sensitive low molecular weight peptide substrates instead of protease gels.

The work described in Chapter 4 set out to generate a cDNA library from mixed stage *P. ovis* mites and screen it with sera from animals immunised with mite extract (Smith et al., 2002), the aim being to identify proteins that are associated with the protective immune response. Five of six positive clones, which were strongly recognised by partially protected vaccinated lambs, encoded myosin-based proteins. As noted earlier (Chapter 4), myosin is regarded as a valid vaccine candidate against *Schistosoma mansoni* infection but has not given useful protection against *S. japonicum*. These observations would suggest that the catchin-myosin protein identified is worthy of evaluation in a vaccine trial. However, myosin is highly immunogenic and it would be necessary to conduct more extensive ELISA analysis to further confirm a correlation of myosin antibody titres with protection before embarking on an expensive protection trial. Despite showing no homology to any protein sequences in the public databases, the relevance of remaining positive, clone Po41, to protection cannot be discounted.

The EST analysis (Chapter 5) identified eight different allergens, including homologues of the house dust mite group 1, 2, 5 and 7 allergens, a fatty acid binding protein, tropomyosin, paramyosin and a heat shock protein. The identification of these allergens greatly expands on previous studies, as only the *P. ovis* group 2 allergen homologue had been identified to date (Temeyer, Soileau, & Pruett, 2002). In addition, the proteases identified in the biochemical analysis and the EST analysis may also be allergens, as it has been noted that the proteolytic function of many of the house dust mite allergens is associated with their allergenicity (Pomés & Chapman, 2001). These proteins were identified either from a random selection of clones from a cDNA library (EST analysis) or from their proteolytic activity (biochemical analyses). Whether they are allergenic in *P. ovis* infestation still

remains to be determined. This could be achieved by carrying out skin test assays using either purified native or recombinantly derived proteins to determine if a hypersensitivity response was induced in sensitised animals. Alternatively, the recombinant proteins could be tested by ELISA for evidence of IgE binding in sera from sensitised animals.

Antioxidants, such as thioredoxin peroxidase, superoxide dismutase and glutathione S-transferase, were also identified from the EST analysis. These enzymes may have a role in protecting the mite from free radicals that are likely to be produced by the host as part of a protective immune response, but there is no experimental evidence, as yet, to confirm this theory. The availability of DNA sequences for these proteins opens up the possibility of quantifying the expression of some of the mite antioxidant proteins by using, for example, real time PCR. The mites could be exposed to increasing concentrations of free radicals *in vitro* and alterations in the levels of antioxidant gene expression determined. This type of analysis could also be conducted on mites harvested from donor sheep as the infestation progresses. If sufficient mites were available, biochemical analyses of enzyme activity in mite extracts could also be carried out. Either way, the aim would be to define whether or not such a response occurred and to define if it could be correlated to events in the manifestation of the disease process.

A number of putative vaccine candidates were also identified, such as cathepsin B, cathepsin L, fatty acid binding protein, paramyosin, tropomyosin, myosin and GST. These proteins have been shown to be protective in other parasitic infections. For example, vaccination with cathepsin B cysteine proteases results in significant protection against *Haemonchus contortus* infection in lambs (Knox, Smith, & Smith, 1999) and cathepsin L proteases are protective against fasciolosis in both sheep (Wijffels et al., 1994) and cattle (Dalton et al., 1996a). Paramyosin is one of the proteins which has been considered for vaccine development against the schistosome, *S. japonicum* (McManus et al., 2002) and myosin also offers some protection against *S. mansoni* infection in mice (Soisson et al., 1992). In addition, immunisation of sheep with tropomyosin results in partial protective immunity against gut nematodes (Cobon et al., 1989). Vaccination with fatty acid binding proteins has been shown to give significant levels of protection against *Schistosoma*

mansoni in mice (Tendler, Brito, Vilar et al., 1996) and *Fasciola hepatica* infection in cattle (Estuningsih, Smooker, Wiedosari et al., 1997). Finally, immunisation with GST has been shown to offer significant levels of protection against *F. hepatica* in both sheep (Sexton et al., 1990) and cattle (Morrison et al., 1996). In the case of *P. ovis* infestation, a vaccine would need to abolish the welfare problem by eliminating the clinical symptoms such as the intense pruritis and hypersensitivity response. In addition, it would have to protect the animals for a relatively long period of time, at least as long as the standard treatments today, and would also have to be relatively cheap in order to compete economically with the acaricides used at present. Furthermore, it may be pertinent to remember that there are many stages during the mite lifecycle when the mites are in quiescence and therefore do not feed. This may be an important consideration when developing both vaccines and new drug targets.

The sequence analysis conducted (Chapter 6) indicated that each of the cysteine proteases had a signal peptide present, which suggests that they may be secreted. Also, a zinc-binding domain is present in the *P. ovis* cathepsin L sequence. A metal binding domain has not previously been reported from cathepsin L proteases, which suggests that this may be a novel class of protease. To determine if this domain is functionally active, it will be necessary to conduct enzyme activity assays in the presence of a chelating agent. If the domain were functionally important then it would be expected that the enzyme activity would decrease in the presence of these agents.

The phylogenetic analysis conducted indicated that the *P. ovis* group 1 allergen homologue is neither a cathepsin B-like nor a cathepsin L-like protease. The group 1 mite allergens were found to form a unique clade, which suggests that they may have specific domains that define them as allergens. These domains may be identified in the future by careful comparisons of protease and allergen protein sequences and perhaps the use of 3D conformational modelling to determine which epitopes are present on the surface of the protein, and therefore could be “seen” by the immune response.

In the future, it may also be interesting to determine when specific proteins are produced in the lifecycle of the parasite. Several other studies have shown that the production of a number of proteins is developmentally regulated and occurs in a

stage-specific manner (Pratt, Cox, Milhausen et al., 1990; Reed, Spithill, Strugnell et al., 1998; Hoekstra et al., 2000). This information may be useful when deciding which proteins to target for either vaccine or drug targets.

In conclusion, the work undertaken in this project has identified that there is a range of protease activities present in sheep scab mites with cysteine proteases dominating but aspartyl and metallo-proteases also present. These data were confirmed by the identification of three types of cysteine protease from the EST analysis. The sequence analysis of the cysteine proteases indicated that the group 1 mite allergens, e.g. *Der p 1*, form a unique clade, suggesting that they may have certain signatures that define them as allergens. In addition, the cathepsin L protease identified from *P. ovis* was found to contain a zinc-binding domain, which has not been reported previously. The *P. ovis* cathepsin L may therefore, represent a new type of cysteine protease. The EST analysis also identified a variety of other proteins, many of which may be important in the pathogenesis of this disease. A number of allergens were identified, all of which require further analysis to determine their roles in the initiation or aetiology of the disease. Many types of free-radical scavenging enzyme were also identified and these may suggest a mechanism by which the mite evades the host immune response. This project has served to extend significantly the knowledge of the sheep scab mites at the molecular level and these data may be useful in the future to define for example, the interactions between mites and hosts or in the search for vaccine candidates.

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APPENDIX 1 – NUCLEOTIDE AND AMINO ACID SEQUENCE OF Po 41

AAAATTCTC	CTTCATAAA	AAAATCAAA	ATGAAATTC	GCCGTTTAC	-45
K I L	L H K	K I K	M K F	A V Y	-15
TTTTTGGTT	GTTGCTGCA	TTTGTAGCC	GTAGCTATT	GCTGATCAA	-90
F L V	V A A	F V A	V A I	A D Q	-30
GCAAAAGAT	CTTCAAAGA	GCTAAAGTA	TTGTTGGAT	CAAGCTAAA	-135
A K D	L Q R	A K V	L L D	Q A K	-45
GAATTGAAA	GATCAAACC	AACGAAGAA	ATTAGCAAA	TTACCAACA	-180
E L K	D Q T	N E E	I S K	L P T	-60
GACAAAAAA	CGATACAAA	GTTGTTAAA	TTTATCTTA	AAACGAGAA	-225
D K K	R Y K	V V K	F I L	K R E	-75
TTGGATGGT	TTAATCTTA	CTTACAACT	AAAATCCAA	GAAATAGTT	-270
L D G	L I L	L T T	K I Q	E I V	-90
ACAAAAGGT	GAAGCAGGC	CAATTAAGT	GATGATGAA	TTGAAAAAA	-315
T K G	E A G	Q L S	D D E	L K K	-105
TTGAAATCA	TATGAAGCT	CAATTATCA	TTGTTGGAA	GCTGCAATT	-360
L K S	Y E A	Q L S	L L E	A A I	-120
GAAGGAGAA	TTGTATTTT	TTGAAATAC	AAAAAACGT	AAACCAACT	-405
E G E	L Y F	L K Y	K K R	K P T	-135
GTTGAAGAT	GATGATGAA	GTATTGTTT	TCCGGTGTT	GCCGATTTA	-450
V E D	D D E	V L F	S G V	A D L	-150
ATGTTTTGA					- 459
M F .					- 152

The nucleotide and amino acid sequences for Po41. The nucleotide is on the upper line, with the amino acid sequence written below. The number at the end of each line refers to the numbers of nucleotides or amino acids respectively.

APPENDIX 2 – PROTEIN SEQUENCES USED FOR PHYLOGENETIC ANALYSIS OF CYSTEINE PROTEASES

The protein sequences used and their GenBank accession numbers, for the phylogenetic analysis of the *P. ovis* cysteine proteases. Refer to Chapter 6, Figure 6.9.

Sequence ID	Organism	Accession Number
ActchCatB	<i>Actidinia chinensis</i>	P00785
Aedegcatb	<i>Aedes aegypti</i>	L41940
Anncacpro	<i>Anchylostoma caninum</i>	U02611
Anccacb1	<i>Ancylostoma caninum</i>	U18912
Anccacb2	<i>Ancylostoma caninum</i>	U18911
Ascsucab	<i>Ascaris suum</i>	U51892
Caecelcpr	<i>Caenorhabditis elegans</i>	U11245
Caecelcpr3	<i>Caenorhabditis elegans</i>	P43507
Caecelcpr4	<i>Caenorhabditis elegans</i>	P43508
Caecelcpr5	<i>Caenorhabditis elegans</i>	P43509
Caecelcpr6	<i>Caenorhabditis elegans</i>	P43510
Caecelcys1	<i>Caenorhabditis elegans</i>	P25807
Celf15d4	<i>Caenorhabditis elegans</i>	Z80344
Celf32b5	<i>Caenorhabditis elegans</i>	AF003148
Celf41e6	<i>Caenorhabditis elegans</i>	AF016448
Celm04g12	<i>Caenorhabditis elegans</i>	Z81103
Celf26e4	<i>Caenorhabditis elegans</i>	Z81070
Celf57f5	<i>Caenorhabditis elegans</i>	Z75953
Carpacari	<i>Carica papaya</i>	P10056
DERF1	<i>Dermatophagoides farinae</i>	P16311
DERP1	<i>Dermatophagoides pteronyssinus</i>	P08176
Dicticys1	<i>Dictyostelium discoideum</i>	P04988
EURM1	<i>Euroglyphus maynei</i>	P25780
Fashefc2	<i>Fasciola hepatica</i>	Z22763
Fashecl1	<i>Fasciola hepatica</i>	U62288
Fashecl2	<i>Fasciola hepatica</i>	U62289
fashecls	<i>Fasciola hepatica</i>	L33772
Gaincpi3	<i>Giardia intestinalis</i>	U83277
Haecopdm4	<i>Haemonchus contortus</i>	Z69345
Haecoac3	<i>Haemonchus contortus</i>	D48435
Haecoac4	<i>Haemonchus contortus</i>	C48435
Haecoac5	<i>Haemonchus contortus</i>	B48435
Haecocp6	<i>Haemonchus contortus</i>	Z81327
Haecocys	<i>Haemonchus contortus</i>	P19092
Haecocys2	<i>Haemonchus contortus</i>	P25793
Haecopdm5	<i>Haemonchus contortus</i>	Z69346

Hetgycatl	<i>Heterodera glycines</i>	Y09498
Hetglcats	<i>Heterodera glycines</i>	Y09499
Homamcys1	<i>Homarus americanus</i>	P13277
Homamcys2	<i>Homarus americanus</i>	P25782
Homamcys3	<i>Homarus americanus</i>	P25784
Horvualeu	<i>Hordeum vulgare</i>	P05167
Horvucys1	<i>Hordeum vulgare</i>	P25249
Humancatc	<i>Homo sapiens</i>	X87212
Humancath	<i>Homo sapiens</i>	P09668
Humancatk	<i>Homo sapiens</i>	P43235
Humancatl	<i>Homo sapiens</i>	P07711
Humancats	<i>Homo sapiens</i>	P25774
Leimecatb	<i>Leishmania mexicana</i>	Z48599
Leimecpa	<i>Leishmania mexicana</i>	P25775
Leimecpb	<i>Leishmania mexicana</i>	P36400
MaizeCys1	<i>Zea mays</i>	Q10716
MaizeCys2	<i>Zea mays</i>	Q10717
Naefocpro	<i>Naegleria fowleri</i>	U42758
Nepnocle	<i>Nephrops norvegicus</i>	S47432
Nepnocls	<i>Nephrops norvegicus</i>	S47433
OncvuCatC	<i>Onchocerca volvulus</i>	U71150
OstoCys3	<i>Ostertagia ostertagii</i>	Q06544
OstoCys1	<i>Ostertagia ostertagii</i>	P25802
Parwmntp	<i>Paragonimus westermani</i>	D21124
PenvapCp1	<i>Penaeus vannamei</i>	S53027
PenvapCp2	<i>Penaeus vannamei</i>	X99730
PovisCathB2	<i>Psoroptes ovis</i>	BQ834913
PovisCathL	<i>Psoroptes ovis</i>	BQ834906
PovisGp1	<i>Psoroptes ovis</i>	BQ834615
ShjpCatC	<i>Schistosoma japonicum</i>	U77932
ShmaCatC	<i>Schistosoma mansoni</i>	Z32531

APPENDIX 3 – PUBLICATIONS ARISING FROM THIS THESIS

Kenyon, F. & Knox, D. P. (2002) The proteinases of *Psoroptes ovis*, the sheep scab mite – their diversity and substrate specificity. *Veterinary Parasitology* **105**: 317-325.

Kenyon, F., Welsh, M., Parkinson, J., Whitton, C., Blaxter, M. L. and Knox, D. P. (2002) Expressed sequence tag survey of gene expression in the scab mite *Psoroptes ovis* – allergens, proteinases and free radical scavengers. *Parasitology* manuscript submitted.



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The proteinases of *Psoroptes ovis*, the sheep scab mite—their diversity and substrate specificity

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Abstract

The sheep scab mite, *Psoroptes ovis*, causes severe dermatitis in infected sheep with severe welfare and production implications. The dermatitis has the characteristics of an immediate hypersensitivity type reaction which, by analogy to other mite species, including the house dust mites (*Dermatophagoides* spp.), is likely to be invoked by a variety of allergens including mite-derived proteinases. Here, the proteinases in *P. ovis* extracts were characterised using substrate gel analysis, inhibitor sensitivity and their ability to degrade a variety of potential natural protein substrates. These analyses showed that mites contain several proteinases which could be differentiated on the basis of molecular size and inhibitor sensitivity with cysteine, metalloproteinases and aspartyl proteinases predominating. These proteinases degraded collagen and fibronectin, possibly indicative of a role in lesion initiation, they degraded several blood proteins, a property which may aid mite feeding and they degraded immunoglobulin G, possibly aiding immuno-evasion. Because proteinases, particularly the cysteine class, are demonstrably allergenic in other mite infestations, these proteinases clearly merit further immunological and biochemical definition. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Sheep-arthropoda; *Psoroptes ovis*; Proteinases

1. Introduction

The mite, *Psoroptes ovis*, is an important ectoparasite infecting sheep, goats and cattle being particularly prevalent in sheep in the UK and in cattle in Europe and the USA. *P. ovis* can induce a severe welfare condition and is highly contagious being spread by direct or indirect contact (Lewis, 1997). Infection provokes an extensive dermatitis, scab formation and intense pruritis (Smith et al., 2001). Infected animals show pronounced behaviour responses including rubbing the area of the scab lesion, scratching, wool loss, wounding

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and fitting (Corke and Broom, 1999). Initial focal lesions spread outwards, coalesce and may extend over the whole body. The host response to infestation has all the characteristics of an immediate type hypersensitivity reaction and this response can be experimentally induced by injection of mite extracts intradermally (Wikel, 1984). The individual antigens which invoke this response remain largely undefined although a 16 kDa allergen has, recently, been identified (Pruett, 1999).

Allergens have been extensively studied in the principal house dust mites *Dermatophagoides pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei* with comparative data from storage mites, such as *Lepidoglyphus destructor* and the tropical mite *Blomia tropicalis* becoming available (Stewart, 1995). These analyses show that each species contains several allergens with considerable inter-species similarities. Several allergens are peptidases and include cysteine proteinases (e.g. *Der p1*; Chua et al., 1988) and serine proteinases (Heymann et al., 1989; Stewart et al., 1989). Other peptidases identified include amino- and carboxypeptidases (Stewart et al., 1986, 1991) but their allergenicity remains to be confirmed (Stewart, 1995). These enzymes are commonly found in mite faecal pellets and may facilitate both intracellular and extracellular digestion, in the case of the latter to aid the penetration of collagen and keratin barriers in the skin (Stewart, 1995).

The present study was initiated to define the range of proteinases present in whole extracts of *P. ovis* and to examine their ability to degrade a range of protein substrates which may be encountered in the host environment.

2. Materials and methods

2.1. Mites and mite extracts

P. ovis mites were harvested from skin sections from sheep infested with mites maintained at the Moredun Research Institute, Edinburgh. Mites were extensively washed and water-soluble (S1) and membrane associated (S2) fractions prepared from approximately 500 mg mites as described by Smith and Smith (1993). These fractions were selected for study as they were found to have protective qualities in vaccination trials (Smith, unpublished data).

2.2. Substrate gel analysis

The S1 and S2 fractions (10 µg) were fractionated in 10% SDS-PAGE gel slabs (Laemmli, 1970) containing 0.1% (w/v) protein substrate. The protein substrates used were gelatin, haemoglobin, azocasein, and fibrinogen (all purchased from Sigma, UK). After electrophoresis at 200 V for 45 min in a Mini Protean® II Dual Slab Cell (BioRad, UK), the gels were washed in four changes of 2.5% Triton X-100 in 1 h to remove the SDS. The gels were then incubated in 0.1 M buffer (pH 3–6, acetate, pH 7–9, phosphate and pH 10–11 glycine-based buffers) with and without the addition of 2 mM dithiothreitol (DTT), for 16 h at 37 °C. DTT is known to activate cysteine proteases, which are generally found at acidic pH levels. Zones of proteolysis were visualised by 0.25% Coomassie blue staining.

2.3. Degradation assays

Fibrinogen, haemoglobin (each 2 mg/ml), IgG (1 mg/ml, purified from sheep serum using protein-G agarose (Boehringer Mannheim)) and fibronectin (1 mg/ml) were dissolved in distilled water (dH₂O). Substrate (10 µl) was mixed with 4 µg of either the S1 or S2 extract or 3 µl of PBS to act as control. Two microlitres of the appropriate pH buffer as described above but here, all at 0.2 M, was then added and the samples incubated for 16 h at 37 °C. After the addition of an equal volume of reducing sample buffer, electrophoresis was carried out at 200 V for 45 min and gels stained with 0.25% Coomassie blue.

The degradation of azocoll, elastin-orcein and keratin-azure (all from Sigma) was sought using a spectrophotometric assay. Briefly, 100 µl of azocoll (10 mg/ml in dH₂O), 1 mg elastin-orcein or 1 mg of keratin-azure was mixed with 100 µl of 0.2 M buffer (as above, pH 3–5 included 2 mM DTT) and either 1.5 µg of the S1 or S2 extract, or 2 µl of PBS to act as the control. Samples were incubated at 37 °C for 16 h, spun briefly at 13,000 × g, and the absorbance of the supernatant determined spectrophotometrically at 520 nm (azocoll), 550 nm (elastin-orcein) or 620 nm (keratin-azure).

2.4. Inhibitor studies

Class-indicative proteinase inhibitors were either added to the overnight incubation buffer for the substrate gels, or included in the degradation assays. Inhibitors used (Sigma, UK) and their final concentrations were: 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF, 2 mM); L-trans-epoxysuccinyl-L-leucylamido- (4-guanidino)-butane (E64, 10 µM); pepstatin A (10 µM) and 1,10-phenanthroline (1 mM).

2.5. Antibody inhibition studies

IgG was purified from sera of vaccinated sheep protected against challenge, from sheep with a natural infestation of *P. ovis* or from naive animals by a method described previously (Young et al., 1995). S1 or S2 extract (10 µg) was mixed with 1 µl dH₂O to act as a control or 1, 5 or 10 µg of the purified IgG and incubated at 37 °C for 1 h prior to electrophoresis in 5% native gels containing 0.1% gelatin. The gels were then incubated overnight at 37 °C in pH 5 acetate buffer with or without the addition of 2 mM DTT and stained in 0.25% Coomassie blue.

3. Results

3.1. Substrate gel analysis

Proteinase activity, some of which, at acidic and neutral pH, was enhanced by the presence of DTT, was visible over the pH range 3–11 with gelatin as substrate (Fig. 1). The broad size range where proteolysis was visible (35 to >205 kDa) appeared to comprise several poorly resolved zones (see pH 4 + DTT), the bulk of which were almost completely inhibited by the cysteine proteinase inhibitor, E64 (Fig. 2A). Two sharp zones of proteolysis at

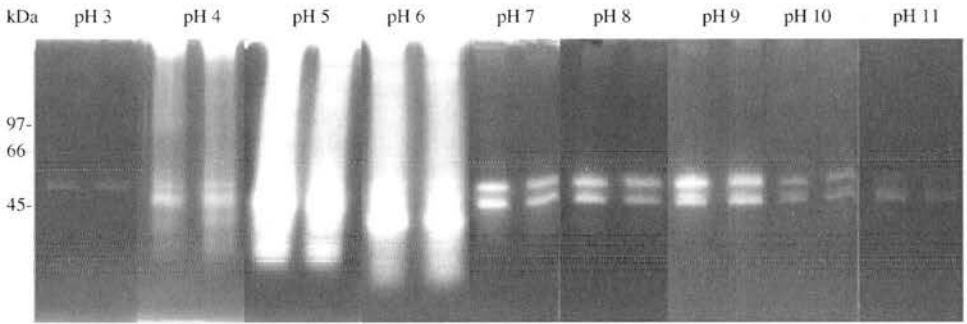


Fig. 1. Gelatin-substrate gel. S1 extract is to the left of each strip, S2 to the right. The pH 3–6 had 2 mM DTT added to overnight incubation buffer.

approximately 50 and 55 kDa were evident over a broad pH range (pH 4–11) with optimal activity at pH 6. These bands were fairly insensitive to the inhibitors tested but activity was reduced by the metalloproteinase inhibitor 1,10-phenanthroline at pH 9 (see Fig. 2B).

When azocasein was used as substrate in the gels, activity was very much reduced compared to gelatin with only faint bands at 45 and 66 kDa being evident, both sensitive to E64, in both extracts at pH 6 and 7 (not shown). No activity was observed with haemoglobin as substrate. Proteolysis of fibrinogen (Fig. 3) was comparatively weak and only visible at pH 5 and 6. However, additional activity, not evident with gelatin as substrate, was observed at 44 and 38 kDa. Activity was strongest in the membrane associated S2 mite extract and was insensitive to 1,10-phenanthroline.

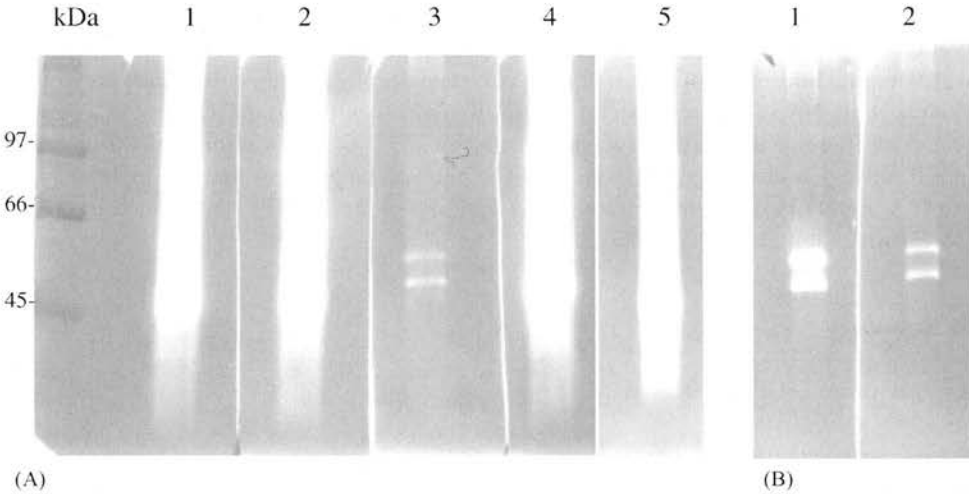


Fig. 2. Gelatin-substrate gel. Panel (A): pH 5 plus 2 mM DTT. Lane 1: control mite extract alone, 2: plus 2 mM AEBSF, 3: plus 10 µM E64, 4: plus 10 µM pepstatin, 5: plus 1 mM 1,10-phenanthroline. Panel (B): pH 9. Lane 1: control mite extract alone, 2: plus 1 mM 1,10-phenanthroline.

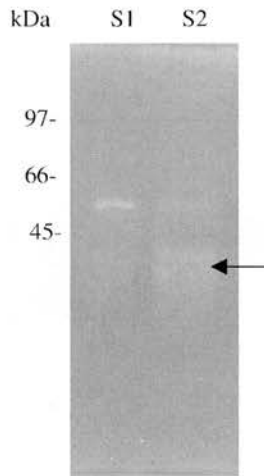


Fig. 3. Fibrinogen-substrate gel, pH 6, of S1 and S2 extracts without DTT, showing the presence of zones of proteolysis (arrow) at approximately 40 and 38 kDa, not observed in gelatin gels.

3.2. Degradation assays

No differences were observed when degradation with S1 and S2 extracts were compared. Results shown were with pooled S1 and S2.

3.2.1. Haemoglobin

Assays with haemoglobin, fibrinogen, IgG and fibronectin are shown in Fig. 4. Haemoglobin degradation was optimal at pH 3 and completely inhibited by the aspartyl proteinase inhibitor, pepstatin. It was unaffected by other class-indicative inhibitors, most notably E64 (Fig. 4A).

3.2.2. Fibrinogen

All three fibrinogen peptide chains were completely degraded at pH 4 and 5 with no degradation evident above pH 5. Degradation was abolished by pepstatin and partially reduced by 1,10-phenanthroline (Fig. 4B).

3.2.3. IgG

IgG degradation was restricted to the heavy chain (~ 55 kDa) and only evident at pH 4 and 5, resulting in a product at about 30 kDa (Fig. 4C). Degradation was completely inhibited by 1,10-phenanthroline and E64 and slightly inhibited by pepstatin and AEBSF.

3.2.4. Fibronectin

Fibronectin was degraded across a broad pH range from pH 3 to 9. At pH 5, proteolysis was inhibited by E64 and 1,10-phenanthroline (Fig. 4D), by pepstatin at pH 3 and slightly inhibited by both E64 and pepstatin at pH 9 (not shown).

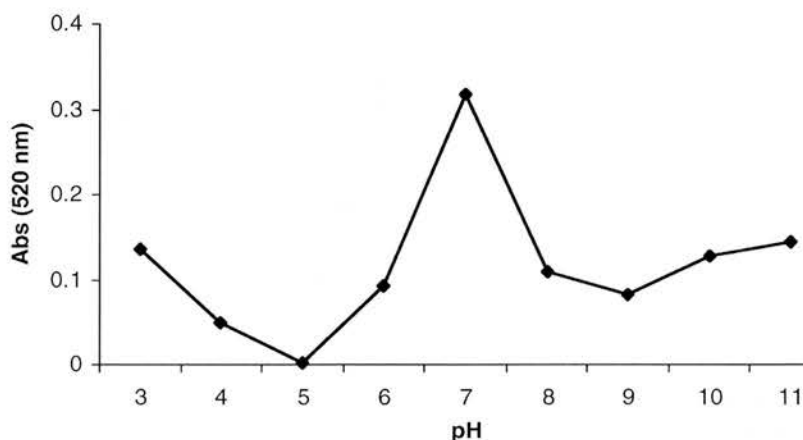


Fig. 5. Azocoll degradation assay showing the pH optima. Each data point on the graph consists of the absorbance at each pH level, minus the absorbance of a PBS control at the equivalent pH level.

3.2.5. Azocoll, elastin-orcein and keratin-azure

Azocoll was degraded by the mite extracts with optimal activity at pH 7 (Fig. 5), this only being inhibited by 1,10-phenanthroline.

Keratin-azure and elastin-orcein were not degraded by the mite extract.

3.3. Antibody inhibition assays

Antibody interactions with the extract proteinases were sought on the basis that antibody binding to the protease would retard migration in gelatin-substrate gels (Young et al., 1995). There were no differences observed between the control S1 and S2 extracts and those that had been pre-incubated with antibody purified from either the vaccinated and protected sera, control sera or the natural infestation sera (results not shown).

4. Discussion

Previous studies (Nisbet and Billingsley, 1999, 2000) suggested that the major endopeptidase activity in *P. ovis* was attributable to an aspartyl proteinase optimally active at low pH and capable of degrading haemoglobin. In addition, Nisbet and Billingsley (2000) showed that *P. ovis* extracts contained a 29 kDa cysteine proteinase using a biotinylated probe. The present study considerably expands these observations showing that extracts of *P. ovis* contain a range of proteinases with the ability to degrade a variety of host proteins. Importantly, we show that *P. ovis* extracts may contain several different cysteine proteinases which may be counterparts of important allergens already identified in house dust and storage mites and may play a central role in lesion development.

At acidic pH, cysteine proteinase activity predominated (Fig. 1), as indicated by E64 inhibition (Fig. 2A). These enzymes degraded the soluble collagen derivative, gelatin,

and fibronectin, an extracellular matrix glycoprotein component of basement membranes (Fig. 4D). They did not degrade keratin or elastin. This suggests that keratin is not a major food source for *Psoroptes* in contrast to house dust mites where skin squames containing keratin are thought to be a major food source (Stewart et al., 1991). Metalloproteinases, indicated by 1,10-phenanthroline inhibition (Fig. 2B), also degraded gelatin. Clearly, proteolysis of these substrates may facilitate penetration of the skin barrier and lesion initiation.

Mite proteinases may perform an anticoagulant function as judged by fibrinogen degradation attributable to aspartyl and metalloproteinases. This action would prolong serous exudation from the lesion and, presumably, aid mite feeding. In agreement with the observations of Nisbet and Billingsley (1999), haemoglobin degradation was solely attributable to aspartyl proteinase activity. Both the cysteine and metalloproteinases played a role in immunoglobulin G degradation with this being confined to the heavy chain, expanding the observations of Pettit et al. (2000). This restricted digestion of the heavy IgG chain suggests that activity is unlikely to be simply the non-specific outcome of mixing protein and proteinases and may indicate that immunoglobulin degradation could enhance mite survival in vivo.

The dust mites *Dermatophagoides* spp. produce a range of allergens with different biological activities (Stewart, 1995). In particular, the group 1 allergens *Der p1* and *Der f1* are cysteine proteinases found in mite whole body and faecal extracts, are synthesised by cells lining the gastro-intestinal tract (Thomas et al., 1991) and may degrade skin collagens and keratin (Colloff, 1993). The cysteine proteinases described here may include a *P. ovis* equivalent of the group 1 allergens. A recent expressed sequence tag (EST) survey of genes expressed by *P. ovis* (Kenyon et al., unpublished) indicated that the most abundant cysteine proteinase showed 69% amino acid sequence identity with *Der p1*. This study identified several other distinct cathepsin B and L-type cysteine proteinases encoding transcripts as well as a variety of matrix metalloprotease homologues. The present study draws attention to the potential importance of metalloproteinases in mite physiology and these are clearly worthy of further analysis. Of further note, the group 3, 6 and 9 allergens of *Dermatophagoides* spp. are all serine proteinases and some, especially in the case of *D. pteronyssinus*, show a high frequency of reactivity in affected individuals (Stewart, 1995). Here, no evidence for serine proteinase activity in *P. ovis* extracts was provided despite the use of a variety of substrates and proteinase class-indicative inhibitors.

There is now a requirement to define (1) if these proteinases illicit an immune response in the course of infection, (2) if any invoke an IgE response which would implicate them in the hypersensitivity response to the mite and (3) if any are associated with protective immunity. In the present study, we addressed the latter by seeking antibody/proteinase interactions with a gel-based technique previously used with success in the definition of proteinases in the excretory/secretory components of *Teladorsagia circumcincta*, the ovine abomasal nematode (Young et al., 1995). Here, antibody was harvested from sheep immunised against infection by repeated natural challenge or by vaccination with mite extracts, then reactivity with mite extract proteinases compared with antibody from uninfected controls. No differences were observed in electrophoretic mobility or enzyme activity, suggesting that proteinase inhibition by antibody may not be associated with the protective immune response in *P. ovis* infestation. Work is now in progress to define if recombinant enzyme proteins, expressed in *Escherichia coli*, invoke IgG and/or IgE responses in infected sheep.

Acknowledgements

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Expressed sequence tag survey of gene expression in the scab mite *Psoroptes ovis* – allergens, proteases and free-radical scavengers

proteases

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PROOF

c/

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SUMMARY

Psoroptes ovis, the causative agent of sheep scab, is an important ectoparasitic mite infecting sheep, goats and cattle. Infection is characterized by an extensive dermatitis, scab formation and intense itching. Initial focal lesions spread outwards, coalesce and may extend over the whole body. The host response to infestation has all the characteristics of an immediate-type hypersensitivity reaction but the mite antigens and allergens which initiate this response are almost completely undefined. Here, 507 randomly selected cDNAs derived from a mixed population of *P. ovis* were sequenced and the resultant nucleotide sequences subjected to Cluster analysis and Blast searches. This analysis yielded 280 clusters of which 49 had >1 sequence with 24 showing significant Blast X homology to another protein in the databases. There were 231 sequences which appeared on one occasion and 109 of these showed significant Blast X homology to other sequences in the databases. This analysis identified homologues of 9 different types of allergens which have been characterized in other allergic conditions such as responses to house dust mites. It also identified a number of cysteine proteases which may contribute to lesion development as well as several free-radical scavenging enzymes which may protect the mite from host immune effector responses.

Key words: sheep scab, *Psoroptes ovis*, expressed sequence tag, allergens.

INTRODUCTION

Psoroptes ovis, the causative agent of sheep scab, is an important ectoparasitic mite infecting sheep, goats and cattle. The condition is particularly prevalent in sheep in the UK and cattle in Europe and the USA and is highly contagious being spread by direct or indirect contact. Infection is characterized by an extensive dermatitis, scab formation and intense itching and infected animals show pronounced behaviour responses including rubbing the area of the scab lesion, scratching, wool loss, wounding and fitting. Initial focal lesions spread outwards, coalesce and may extend over the whole body.

The host response to infestation has all the characteristics of an immediate-type hypersensitivity reaction and this response can be experimentally induced by injection of mite extracts intradermally (van den Broek *et al.* 2000). To date, the individual antigens which invoke the characteristic response in sheep scab remain largely undefined. Immunoblots of mite extracts have been probed with sera from immune

or infested sheep. Between 6 and 30 antigens were detected but their nature was undefined (e.g. Boyce & Brown, 1991). Another study identified a 16 kDa allergen which is a mite group 2 allergen homologue (Pruett, 1999; Temeyer, Soileau & Pruett, 2002). Biochemical analyses have shown that the mites contain cysteine, metallo- and aspartyl proteases (Nisbet & Billingsley, 1999, 2000; Kenyon & Knox, 2002). These enzymes can degrade proteins such as collagen, fibronectin, haemoglobin and fibrinogen, suggesting a role in mite feeding and lesion formation (Kenyon & Knox, 2002).

Allergens have been extensively studied in the house dust mites *Dermatophagoides pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei* with comparative data from storage mites such as *Lepidoglyphus destructor* and the tropical mite *Blomia tropicalis* (Stewart, 1995). Each species contains several allergens with considerable interspecies similarities. The allergens are often proteases (peptidases) such as cysteine proteases (e.g. Der p 1; Chua *et al.* 1988) and serine proteases (Stewart *et al.* 1992). Other peptidases identified include amino- and carboxypeptidases (Stewart *et al.* 1992) but their allergenicity remains to be confirmed (Stewart, 1995). These enzymes are commonly found in mite faecal pellets and may facilitate both intracellular

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Table 1. Summary of the EST dataset showing those ESTs encoding proteins with similarity to allergens, proteases, free-radical scavenging enzymes and others of general interest

(For the clusters comprising more than 1 EST, the accession number of the first EST in the cluster is shown. All ESTs can be viewed at <http://nema.cap.ed.ac.uk/Chelicerates/ChelDB.php>.)

Description and Accession no.	No. of EST's	Gene name	Putative function	Homologue in	Blast X E-value	ORF length (aa)	Homology (%)	
							Identity	Similarity
Allergens								
BQ834604	20	<i>Pso-o-II</i>	Group II allergen	<i>P. ovis</i>	1.2 e ⁻⁷²	160	99	99
BQ834615	9	<i>Pso-derF A-1</i>	Secreted cysteine protease	<i>Dermatophagoides farinae</i>	2.1 e ⁻⁷³	231	69	79
BQ834608	4	<i>Pso-gst-2</i>	Glutathione S-transferase	<i>Drosophila melanogaster</i>	9.8 e ⁻²¹	141	45	65
BQ834621	4	<i>Pso-fabp-1</i>	Fatty acid binding protein	<i>Blomia tropicalis</i>	8.8 e ⁻²⁰	134	35	53
BQ834778	3	<i>Pso-gp7-1</i>	Unknown, group 7 allergen	<i>Lepidoglyphus destructor</i>	1.2 e ⁻²²	178	35	57
BQ834975	2	<i>Pso-der p V-1</i>	Der p V IgE binding allergen	<i>D. pteronysinus</i>	8.0 e ⁻³⁵	154	40	64
BQ834874	1	<i>Pso-tropo-1</i>	Tropomyosin	<i>D. farinae</i>	7.6 e ⁻⁹⁴	212	98	98
BQ834786	1	<i>Pso-paramy-1</i>	Paramyosin	<i>Sarcoptes scabiei</i>	1.5 e ⁻⁵⁸	172	90	93
BQ835043	1	<i>Pso-hsp-1</i>	Heat shock protein	<i>Caenorhabditis elegans</i>	9.2 e ⁻¹³	149	41	58
Cysteine proteases								
BQ834615	9	<i>Pso-der F A-1</i>	Secreted cysteine protease	<i>D. farinae</i>	2.1 e ⁻⁷³	231	69	79
BQ834710	1	<i>Pso-cathb-1</i>	Cathepsin B-like cysteine protease	<i>Bombyx mori</i>	1.5 e ⁻⁴⁹	204	48	63
BQ834913	1	<i>Pso-cathb-2</i>	Cathepsin B-like cysteine protease	<i>C. elegans</i>	6.1 e ⁻¹⁶	57	72	84
BQ834906	1	<i>Pso-cathl-1</i>	Cathepsin L-like cysteine protease	<i>Sitophilus zeamais</i>	1.3 e ⁻⁸²	239	65	76
Free-radical scavenging enzymes								
BQ834608	4	<i>Pso-gst-2</i>	Glutathione S-transferase	<i>D. melanogaster</i>	9.8 e ⁻²¹	141	45	65
BQ834873	1	<i>Pso-tpx-1</i>	Thioredoxin peroxidase	<i>Globodera rostochiensis</i>	9.4 e ⁻⁷³	234	75	87
BQ834914	1	<i>Pso-tpx-2</i>	Thioredoxin peroxidase	<i>Homo sapiens</i>	8.8 e ⁻⁶⁸	210	68	85
BQ834951	1	<i>Pso-tpx-3</i>	Thioredoxin peroxidase	<i>D. melanogaster</i>	5.9 e ⁻⁶²	196	85	87
BQ834990	1	<i>Pso-sod-1</i>	Superoxide dismutase	<i>Oryza sativa</i>	1.2 e ⁻⁴⁹	149	70	79
Others of interest								
BQ834600	19	<i>Pso-lim-1</i>	IgM domain containing protein	<i>C. elegans</i>	5.5 e ⁻²⁷	106	59	73
BQ834722	2	<i>Pso-cpi-1</i>	Cysteine protease inhibitor	<i>Bos taurus</i>	6.3 e ⁻¹⁰	155	42	58
BQ834901	2	<i>Pso-pkc-1</i>	Protein kinase C	<i>Aplysia californica</i>	2.3 e ⁻³⁷	173	48	65
BQ835048	2	<i>Pso-cytoc-1</i>	Cytochrome C oxidase	<i>Homo sapiens</i>	1.3 e ⁻¹⁶	218	26	51
BQ834616	1	<i>Pso-pdi-1</i>	Protein disulphide isomerase	<i>Mus musculus</i>	2.2 e ⁻³⁰	213	62	76
BQ834761	1	<i>Pso-fsp-1</i>	Fungal stress protein	<i>Rhizopus nigricans</i>	4.0 e ⁻⁴⁰	238	43	64
BQ834767	1	<i>Pso-adh-1</i>	adenosylhomocysteinease	<i>C. elegans</i>	2.8 e ⁻⁷¹	188	77	85
BQ834801	1	<i>Pso-prot-1</i>	Proteasome subunit beta type 4 precursor	<i>Xenopus laevis</i>	1.8 e ⁻⁴⁶	238	54	73
BQ834896	1	<i>Pso-ier-1</i>	Lymphocyte IgE receptor	<i>Homo sapiens</i>	3.8 e ⁻¹⁰	251	33	47
BQ834927	1	<i>Pso-cut-1</i>	Rigid cuticular protein	<i>Araneus diadematus</i>	2.8 e ⁻⁰⁹	165	50	55
BQ834991	1	<i>Pso-alg2-1</i>	Programmed cell death protein	<i>Mus musculus</i>	2.3 e ⁻⁵³	184	57	77
BQ835077	1	<i>Pso-aladh-1</i>	Haem biosynthesis enzyme	Fission yeast	8.1 e ⁻³⁷	100	38	54

(Ribosome-associated proteins N = 36).

and extracellular digestion, in the case of the latter to aid the penetration of collagen and keratin barriers in the skin (Stewart, 1995).

The present study was initiated with the aim of rapidly identifying further potential allergens and other proteins of importance to lesion formation during *P. ovis* infestation using expressed sequence tag (EST) analysis. In this approach, sequences derived from randomly selected cDNAs can be used to define the genes expressed by an organism. This method has been used with success in the identification of genes from a variety of parasites including the cattle tick, *Boophilus microplus* (Crampton *et al.* 1998), the ovine gastrointestinal nematode, *Haemonchus contortus* (Hoekstra *et al.* 2000), the filarial nematodes, *Brugia malayi* (Blaxter *et al.* 2002) and *Onchocerca volvulus* (Lizotte-Waniewski *et al.* 2000) and the human hookworm, *Necator americanus* (Daub *et al.* 2000).

MATERIALS AND METHODS

cDNA library preparation and expressed sequence tag generation

P. ovis mites (700 mg, mixed sex) were harvested from the skin of experimentally infected sheep (Smith *et al.* 2002) and contaminating skin debris removed by extensive washing. Messenger RNA was prepared from *P. ovis* mites using an mRNA extraction kit (Stratagene, UK) and a cDNA library was constructed in Lambda TriplEx2 cloning vector according to the manufacturer's instructions (Clontech). The library was plated on *XL-1-Blue* cells and recombinant clones picked at random. The library titre was 1.6×10^6 pfu/ml with 89% recombinant phage as judged by blue/white colour selection. The cDNA inserts of randomly selected clones were amplified by PCR using the universal M13 forward and reverse vector primers. Insert cDNAs > 500 bp (75% of the total) were selected for sequencing. The PCR products were cleaned and sequence analyses conducted using ABI rhodamine dye terminators and an ABI 377 automated sequencer (Daub *et al.* 2000).

Bioinformatics

Sequences were checked, vector and poor quality 3' sequences removed using automated methods applied to the *Brugia* EST project and then compared to the public databases (GenBank non-redundant nucleotide and protein databases and dbEST) using the BLAST algorithms (Altschul *et al.* 1990). Sequences were clustered on the basis of homology using CLOBB v1.0 (Parkinson, Guiliano & Blaxter, 2002). Briefly, CLOBB is an iterative clustering method where sequences are clustered on the basis of BLAST similarity. It tracks cluster-specific events such as merging, identifies 'superclusters' of related

clusters and avoids expansion of chimeric clusters (Parkinson *et al.* 2002). Putative function was assigned where possible, all conducted within the quality control parameters outlined by Daub *et al.* (2000). The cluster analysis is available at <http://nema.cap.ed.ac.uk/Chelicerates/ChelDB.php>. Motif analysis was not conducted except in specific instances, outlined below, where signal peptides or potential glycosylation sites were sought. Multiple sequence alignments were conducted using Clustal X (Thompson *et al.* 1997), phylogenetic trees constructed using Phylip 3.5 with the default parameters and visualized with TreeView (Page, 1996).

RESULTS AND DISCUSSION

This study was initiated with the aim of rapidly identifying further potential allergens and other proteins of potential importance to lesion formation during *P. ovis* infestation. Only a group 2 allergen homologue had been cloned and sequenced from *P. ovis* (Temeyer *et al.* 2002) prior to this study. Despite the limited number of ESTs analysed here, the present study identified 45 ESTs (9% of the total) encoding proteins with similarity to known allergens previously identified in house dust, storage and forage mite species (Table 1). These mite species contain several allergens with interspecies similarities which lead to immunological cross-reactivity. The allergens can be loosely divided into 2 categories, namely those with or without enzyme activity (Stewart, 1995). Many of the former are proteases which are derived from cells lining the gastrointestinal tract of the mite and are present in faecal extracts (Stewart, 1995). Proteases may initiate the lesion and contribute to nutrient provision for the mite. The non-enzymatic allergens include heat shock proteins, fatty acid binding proteins and structural proteins such as tropomyosin (reviewed by Stewart, 1995; Pomes, 2002). A number of ESTs encoded free-radical scavenging enzymes which may help the mite evade or modulate the host immune response. These data emphasize the value of EST analysis, even on a small scale, to identify parasite genes encoding proteins of potential importance in the host-parasite interaction.

In total 507 sequences were clustered, including 23 previously generated *P. ovis* sequences downloaded from Genbank. These formed 280 clusters of which 49 had > 1 sequence with 24 showing significant Blast X homology to another protein in the databases. There were 231 sequences which appeared on one occasion and 109 of these showed significant Blast X homology to other sequences in the databases. The unequivocal definition of highly expressed genes by cluster analysis is not possible because of the small size of the present dataset. However, Daub *et al.* 2000 noted that early patterns of abundance in ESTs from the nematode *Brugia*

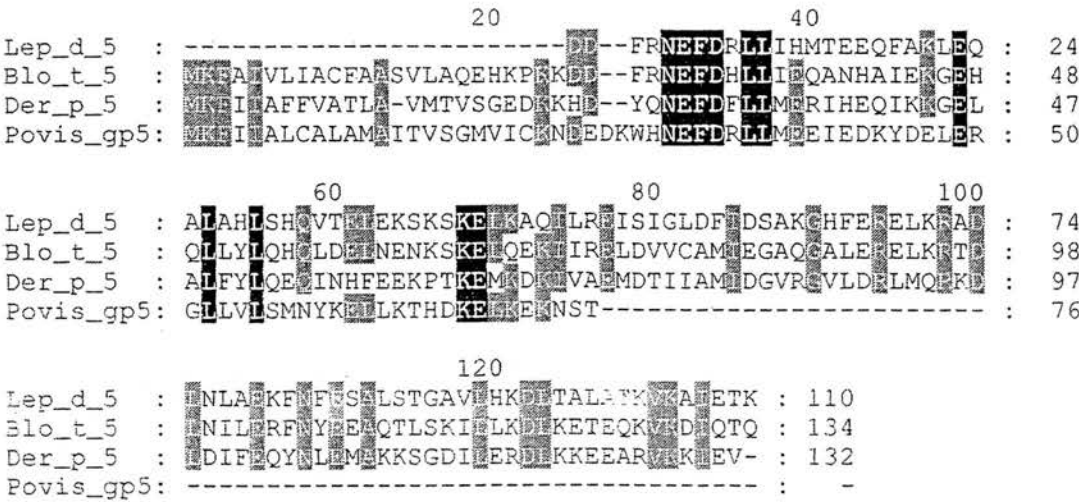


Fig. 1. Alignment of the group 5 allergens. Residues shaded in black are identical in all 4 sequences, those shaded in grey are present in 3 of the 4. -, Gaps inserted to improve the alignment. The sequences used and the GenBank accession numbers are: *Lep d 5*, *Lepidoglyphus destructor*, Q9U5P2; *Blo t 5*, *Blomia tropicalis*, Q96870; *Der p 5*, *Dermatophagoides pteronyssinus*, P14004; *Povis_gp5*, *Pso_Der p V* identified from EST analysis, BQ834975.

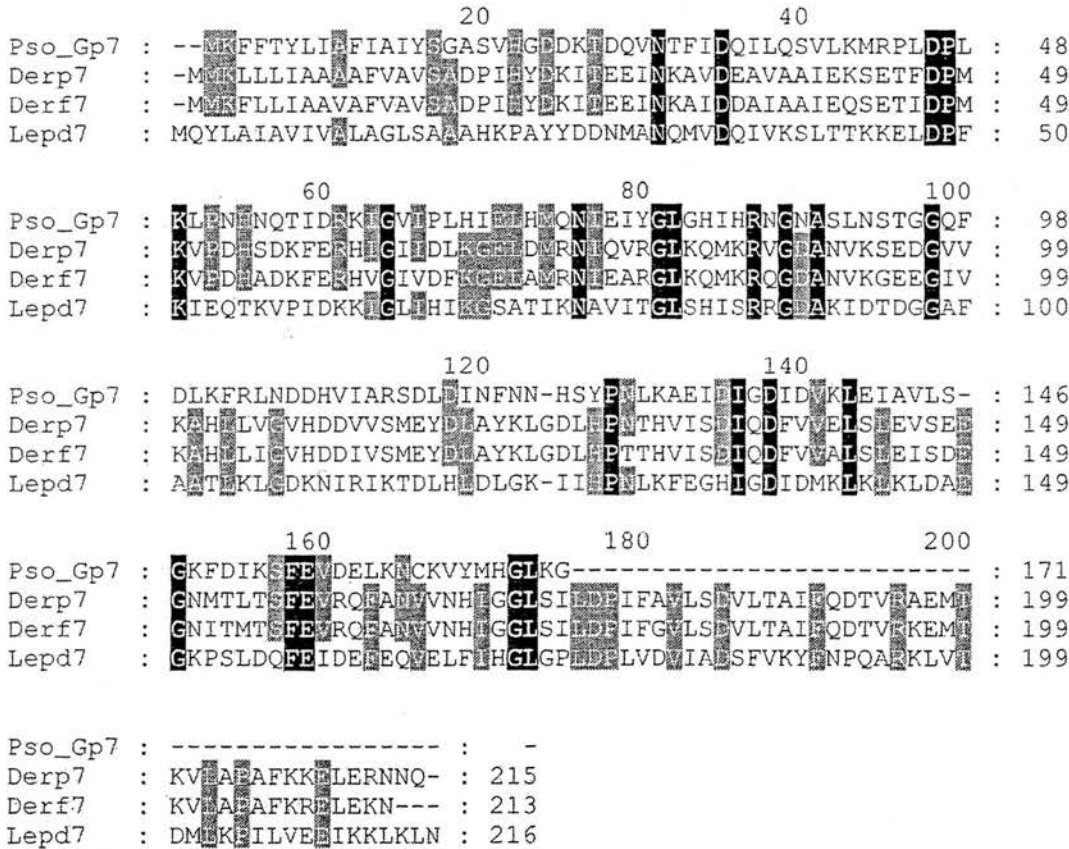


Fig. 2. Alignment of the group 7 allergens. Residues shaded in black are in all 4 sequences, those shaded in grey in 3 of the 4. -, Gaps inserted to improve alignment. Sequences used and their GenBank accession numbers were: *Pso_Gp7*, *P. ovis* group 7 allergen homologue identified from the EST analysis, BQ834778; *Lep d 7*; *Lepidoglyphus destructor*, Q9U1G2; *Der p 7*; *Dermatophagoides pteronyssinus*, P49273; *Der f 7*; *D. farinae*, Q26456.

malayi were, in general terms, confirmed by more extensive sequencing. Nine ESTs encoded homologues of the house dust mite group 1 allergens, which are cysteine proteases (Chua *et al.* 1988). In *Dermatophagoides pteronyssinus*, *Der p 1*, the homologue of *Der F A* in *Dermatophagoides farinae*, is found in high concentrations in mite faeces and is present in the mite gut epithelium (Tovey & Baldo, 1990). Whilst probably functioning as a digestive enzyme, there is now a large body of evidence supporting

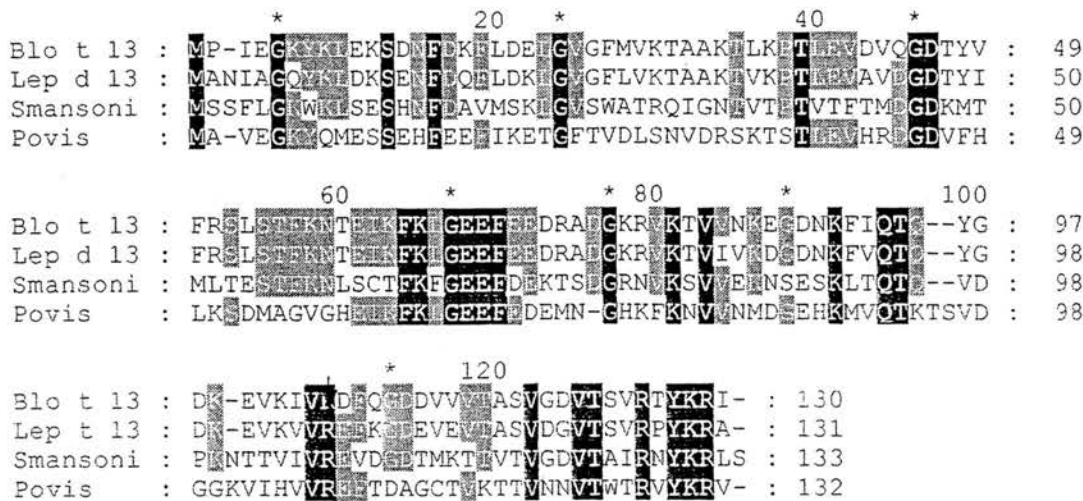


Fig. 3. Alignment of the group 13 (fatty acid binding) allergens. Residues shaded in black are identical, residues shaded in grey are in 3 of the 4. -, Gaps inserted to improve alignment. The sequences used and their GenBank accession numbers as are follows: Blo t 13, *Blomia tropicalis*, Q17284; Lep d 13, *Lepidoglyphus destructor*, Q9U5P1; S. mansoni, *Schistosoma mansoni* fatty acid binding protein, P29498; Povis, *P. ovis* fatty acid binding protein homologue identified from EST analysis, BQ834621. * Glycines involved in β -sheet alignment. ↓ Conserved arginine which participates in electrostatic interaction with the carboxyl group of the fatty acid.

the view that this proteolytic activity contributes to allergenicity (reviewed by Pomes, 2002).

The present study also identified 3 distinct cysteine proteases encoding ESTs (Pso CathB1, Pso CathB2 and Pso CathL1) each represented once and not previously identified in other mite species (Table 1). In helminths, proteases of these types have been implicated in, amongst other functions, digestion and in the penetration of host tissue barriers (Tort *et al.* 1999), some being found in the *in vitro* excretions/secretions indicating a role in extracorporeal digestion. Hence, these proteases may also contribute to the disease syndrome and are worthy of further analysis. An earlier study used various biochemical analyses to demonstrate the presence of aspartyl and metalloproteases in mite extracts and the authors suggested that these enzymes may be important in mite physiology or the aetiology of the disease (Kenyon & Knox, 2002). No ESTs encoding these classes of protease were identified here, a fact which may simply reflect the small number of sequences sampled.

The group 2 allergen sequence recently identified from *P. ovis* (Temeyer *et al.* 2002) was the most abundant in the dataset, with 20 ESTs in the cluster. Sequence analysis of the group 2 allergens from house dust mites suggests they are non-glycosylated proteins which contain leader peptide sequences that are cleaved to yield the mature protein. The mature proteins are approximately 14 kDa and contain 3 disulphide bonds (Nishiyama *et al.* 1993) which are also present in the *P. ovis* homologue (Temeyer *et al.* 2002). These allergens are highly abundant in whole body extracts of the dust mites and are recognized by 80–90% of mite-allergic individuals

(Heymann *et al.* 1989), but have an as yet undefined function in mite physiology. However, Der p 2 has 35% sequence similarity with a human epididymal gene product suggesting a possible role in male reproduction (Mueller, Benjamin & Rule, 1998).

Another cluster of 2 ESTs showed 40% identity with a group V allergen from *Lepidoglyphus destructor*. The Group V allergens are recognized by about 50% of mite-allergic individuals (Tovey *et al.* 1989). These allergens have been identified from *D. pteronyssinus* and *B. tropicalis* but a homologue in *D. farinae* mites has not yet been identified. These allergens range in molecular weight from 14 to 17 kDa and their function is, as yet, unknown. Signal peptide analysis indicates a possible signal peptide cleavage site between positions 18 and 19: VSG-MV. The alignment (Fig. 1) indicates 1 region of close homology between all 4 sequences (residues 31–37, *P. ovis* numbering). Searches using a variety of protein domain servers available on the web failed to ascribe any functional significance to this region.

A cluster comprising 3 ESTs showed closest homology to an allergen, *Lep d 7*, secreted from the storage mite *L. destructor* (Eriksson *et al.* 2001). This allergen is a close homologue of the group 7 allergens from *Dermatophagoides* spp. (Fig. 2). Their biological function is undefined; however, they are known to range from 26 to 31 kDa in size and bind IgE in 50% of sera from ~~human~~ allergic people (Shen *et al.* 1993). The coding sequences for these proteins contain leader peptides indicative of secretion as well as a predicted N-linked glycosylation site (Shen *et al.* 1993). The *P. ovis* sequence contains 4 potential N-linked glycosylation sites, none of which are conserved with other Type 7 allergens, as well as

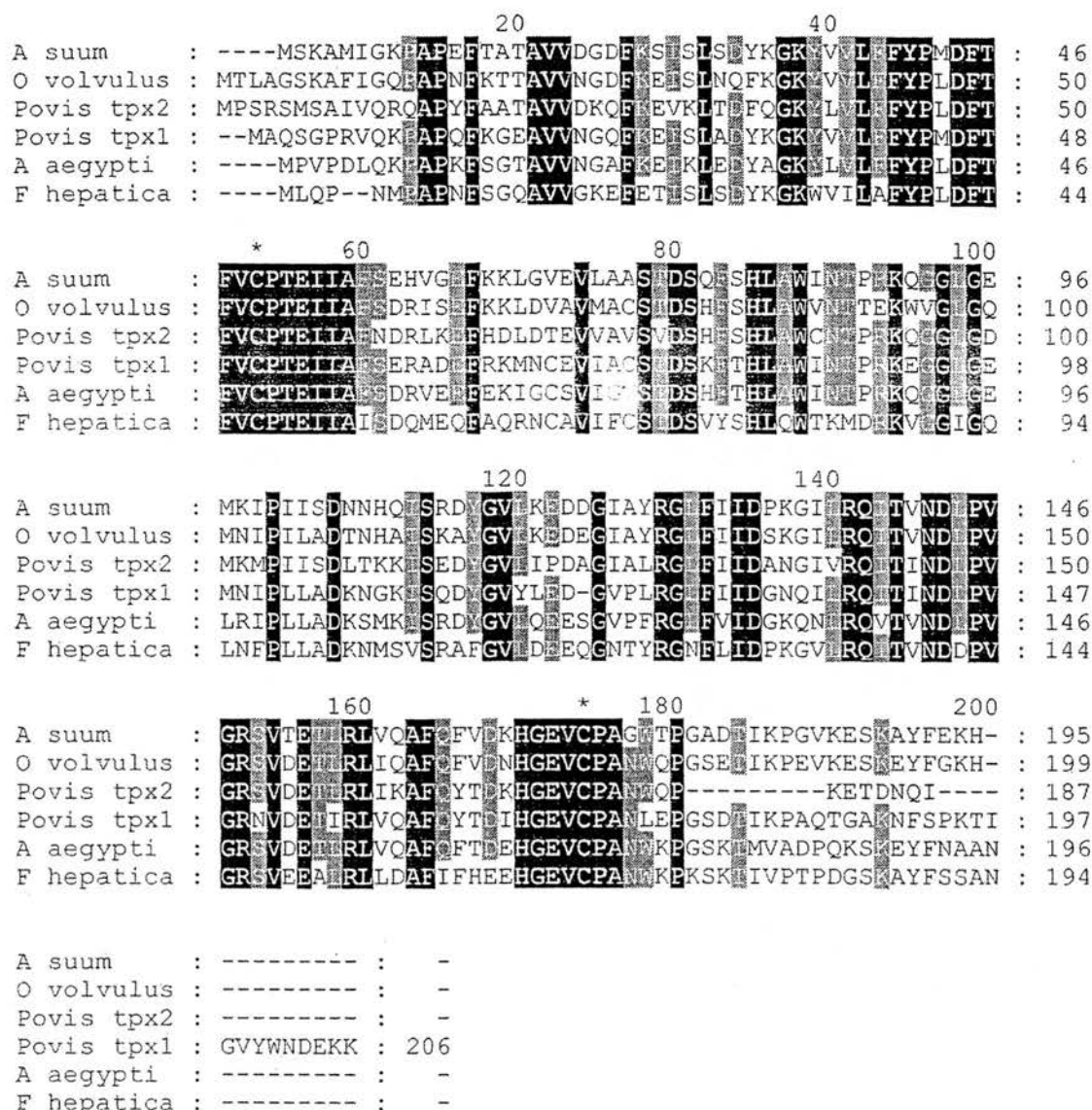


Fig. 4. Alignment of the thioredoxin peroxidase protein sequences. Residues shaded in black are present in all the sequences, those shaded in grey in 5 of the 6. -, Gaps inserted to improve alignment. * Conserved cysteines which contribute to homodimer formation. Sequences used and their GenBank accession numbers are: *Ascaris suum*, Q9NL98; *Onchocerca volvulus*, O44366; *Aedes aegypti*, Q8WSF6; *Fasciola hepatica*, P91883. *Psoroptes ovis* tpx 1 and 2 were identified from the EST analysis, BQ834873 and BQ834914, respectively.

a predicted signal peptide cleavage site at amino acid 22.

Four ESTs encoded a homologue of a mite group 13 allergen, *Blo t* 13, a fatty acid-binding protein (FABP), isolated from the mite *B. tropicalis* (Fig. 3, Caraballo *et al.* 1997). The group 13 allergens have also been identified from the dust mites *Acarus siro* (Eriksson *et al.* 1999) and *L. destructor* (Eriksson *et al.* 2001), and bind IgE in only 11% of sera from mite allergic individuals (Caraballo *et al.* 1997). FABPs are involved in general lipid metabolism acting as intracellular transporters of hydrophobic metabolic intermediates and as carriers of lipids between membranes. Fatty acid binding proteins have been identified in the midgut of larval *Manduca sexta*, the tobacco hornworm (Smith *et al.* 1992),

possibly indicative of a function in lipid uptake from the gut. The *P. ovis* sequence does not contain a putative signal peptide cleavage site indicating it is not secreted but contains a potential *N*-linked glycosylation site at amino acids 122–125. Six of seven glycine residues involved in the β -sheet orientation of fatty acid binding proteins (Sacchettini, Banaszak & Gordon, 1992) are conserved in the *P. ovis* sequence (* Fig. 3) and an arginine (arrowed in Fig. 3), which participates in electrostatic interaction with the carboxyl group of the fatty acid (Sacchettini *et al.* 1992), is conserved in all the sequences in the alignment.

Three other putative allergens, namely tropomyosin, paramyosin and a heat shock protein, were present as single ESTs (Table 1). Tropomyosin is

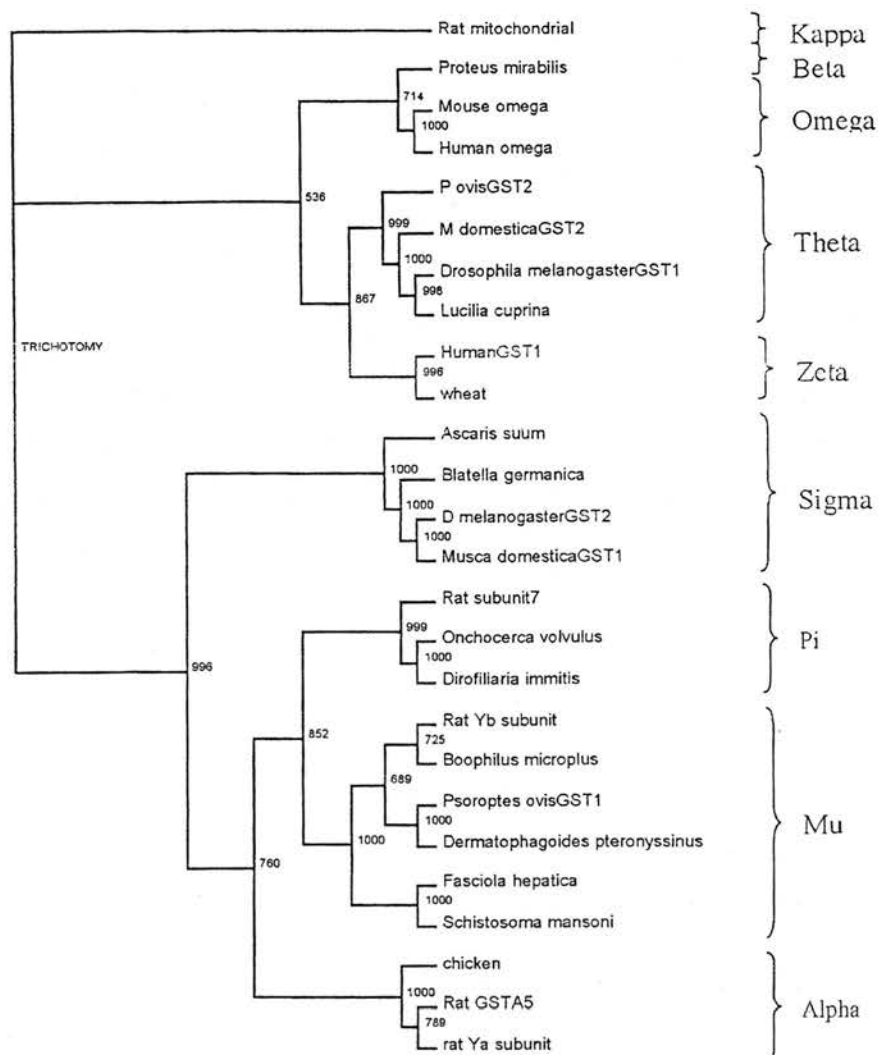


Fig. 5. Phylogenetic analysis of GST protein sequences. Annotation to the right of the diagram denotes the class of GST. The bootstrap values are indicated at the nodes of the tree. The GenBank accession numbers are as follows: Rat mitochondrial, P24473; *Proteus mirabilis*, P15214; Mouse omega, U80819; Human omega, AF212303; *P. ovis* GST2, GST homologue identified in the EST analysis, BQ834608; *Musca domestica* GST2, P46431; *Drosophila melanogaster* GST1, P20432; *Lucilia cuprina*, P42860; Human GST1, AAB96392; Wheat AAD09190; *Ascaris suum*, CAA53218; *Blatella germanica*, O18598; *D. melanogaster* GST2, P41043; *M. domestica* GST1, AAA03434; Rat subunit 7, P04906; *Onchocerca volvulus*, AAA53575; *Dirofilaria immitis*, P46426; Rat Yb subunit, P04905; *Boophilus microplus*, AAD15991; *P. ovis* GST1, AAF19264; *Dermatophagoides pteronyssinus*, P46419; *Fasciola hepatica*, P56598; *Schistosoma mansoni*, P35661; chicken, Q08393; Rat GSTA5, A54858; Rat Ya subunit, P00502.

found in most metazoans and is a structural protein that is involved in the regulation of calcium flux in muscles. Tropomyosin (Der f 10) has been isolated from *D. farinae* (Aki *et al.* 1995) and binds IgE in about 80% of mite allergenic individuals. Paramyosin has been assigned as a Group 11 allergen and has a relatively high molecular weight (~98 kDa). It is recognized by greater than 80% of sera from mite allergic individuals (Tsai *et al.* 1998). Paramyosin is one of the primary candidate proteins for vaccine development against schistosomiasis (McManus *et al.* 2001) and tropomyosin induces partial protective immunity in sheep against gastrointestinal nematodes (Cobon *et al.* 1989).

The heat shock protein identified in this study was homologous to the small heat shock protein family

from *Caenorhabditis elegans*. Heat shock proteins (hsp) are a highly conserved family of proteins that are present in both pro- and eukaryotes and are thought to have play a role in protein folding.

Three types of free-radical scavenging enzyme were identified in this study, namely superoxide dismutase (SOD), thioredoxin peroxidase (TPX) and glutathione S-transferase (GST). Free-radical scavenging enzymes neutralize potentially toxic free-radicals such as the superoxide anion, hydrogen peroxide and the hydroxyl radical (Henkle-Dührsen & Kampkötter, 2001). The presence of the free-radical scavenging enzymes in parasitic organisms has been linked with immunomodulation.

The SOD identified here was homologous to the copper/zinc form of the enzyme. It lacked a

predicted signal peptide indicating it is a cytosolic and is unlikely to be involved in the pathogenesis of the disease.

The thioredoxin peroxidases or peroxidoxins are a family of antioxidant enzymes which have only recently been identified and are characterized by having no cofactors (i.e. metals) (McGonigle, Curley & Dalton, 1997). These enzymes primarily neutralize hydrogen peroxidase thus preventing a build-up of the toxic hydroxyl radical. TPX has not been identified previously in arthropod parasites. Here 3 TPX ESTs were identified, 2 being essentially identical (Pso-Tpx1 and Pso-Tpx3), the third (Pso-Tpx2) being quite distinct (Fig. 4). None of these sequences contained a predicted signal peptide cleavage site indicating they are not secreted. There are 2 main forms of TPX (Henkle-Dührsen & Kampkötter, 2001), the 1-Cys and the 2-Cys types and those identified were the 2-cys type. These enzymes are characterized by the presence of a conserved FVCP sequence around the first cysteine, and a second conserved residue in the C-terminal region, separated from the first by approximately 120 residues (Henkle-Dührsen & Kampkötter, 2001). These conserved cysteines contribute to homodimer formation and function. The presence of more than 1 distinct TPX in the EST dataset suggests that these enzymes may be part of a multi-gene family and may be important in mite physiology.

Glutathione S-transferases (GST) are ubiquitous detoxifying enzymes which catalyse the conjugation of reduced glutathione with a number of electrophiles. In helminths, GSTs usually occur as several isoforms and play a central role in the parasite detoxification system including host-derived anti-parasite factors (Brophy & Pritchard, 1994). There are 9 classes of cytosolic GST defined by a number of factors including substrate specificity, sequence-similarity, and phylogenetic analyses. A GST homologue from *D. pteronyssinus* has been shown to be allergenic (O'Neill *et al.* 1995) and forms group 8 mite allergens. These allergens are approximately 26 kDa in size, and the recombinant molecule is recognized by approximately 40% of mite allergic individuals (O'Neill, Donovan & Baldo, 1995). A mu-class GST homologue was recently identified in *P. ovis* (*P. ovis* GST1) (Lee *et al.* 2002). A theta-class GST, *P. ovis* GST2, was identified in the ESTs (Fig. 5).

A large proportion (47.5%) of the ESTs were not significantly similar to any protein in the public databases and were classed as novel sequences. Of the 23 clusters that encoded novel proteins, 6 comprised 8 or more ESTs suggesting relatively abundant mRNAs and thus protein products. Of these six clusters, 4 had predicted signal peptides suggesting that they could be secreted by the mites and therefore be important in the pathogenesis of the disease. Other proteins of potential interest identified included

a cystatin (a cysteine protease inhibitor), a LIM domain containing protein which may be involved in myogenesis and a homologue of a lymphocyte IgE receptor, the latter being involved in IgE production and the differentiation of B-cells.

In summary, this study has identified several ESTs encoding putative proteins, including allergens, proteases and free-radical scavenging enzymes, which are likely to contribute to lesion formation and disease pathogenesis. These gene products are worthy of further investigation including detailed immunological analyses and possible vaccination studies given that lesion size and mite numbers can be reduced by vaccination of sheep with mite extracts prior to challenge infection (Smith *et al.* 2002).

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